Nitrogen Deposition and Elevated CO_2 Effects on Geochemical Biomarkers in Soil Fractions

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SUMMARY

Anthropogenic activities are globally changing the biogeochemical cycles of carbon (C) and nitrogen (N), because of increasing emissions of carbon dioxide (CO_2) and reactive N into the atmosphere. The main sources are burning of fossil fuels and fertilizer applications. Globally, soils store the largest amount of terrestrial C and annually exchange high quantities of C with plants and the atmosphere. However, under the current anthropogenic environmental changes it is not clear if soils act as sources or sinks of C. It is therefore a major challenge to study the effects of elevated atmospheric CO_2 concentrations and N deposition on the stabilization and turnover of organic matter (OM) in plants and soils.

Stabilization mechanisms of soil OM have recently been investigated using soil density fractionation to separate distinct soil fractions that conceptually relate to different stabilization mechanisms. Stable C isotope analysis ($\delta^{13}C$) of biomarkers in plant biomass and soil fractions offers the possibility to trace OM dynamics at the molecular scale and to distinguish the sources of plant- and microbial-derived OM. The aim of this dissertation is to investigate the effects of elevated atmospheric CO_2 concentrations and N deposition on the stabilization and turnover of plant- and microbial-derived OM in plant biomass and soil fractions.

Model forest ecosystems were established with beech and spruce trees on an acidic soil and treated with ambient and elevated CO_2 concentrations in combination with two levels of N deposition for four years. ^{13}C-depleted CO_2 was used to distinguish between “new” (experimental-derived) and “old” (pre-experimental) OM. Stable C isotope analysis ($\delta^{13}C$) of plant- (long-chain fatty acids) and microbial-derived (amino sugars) biomarkers was performed in above- and belowground biomass of the two tree species and in soil density fractions.

Total organic C and microbial biomarkers (bacterial and fungal) were mainly stabilized by association with soil minerals. Bacterial biomarkers were relatively enriched at soil minerals compared to fungal biomarkers. In contrast, plant biomarkers did not accumulate at soil minerals, which suggest that they are not as effectively stabilized by association with soil minerals as total organic C or microbial biomarkers. These results indicate that stable OM in soils is mainly of microbial origin and preferentially stabilized at soil minerals.

Plant and fungal biomarkers showed equal turnover compared to total organic C, while bacterial biomarkers had slightly lower turnover. These results suggest that soil OM from different origins has generally a similar turnover, independent of its chemical structure and

that soil OM turnover is instead controlled by processes like aggregation or interaction with soil minerals that protect OM from microbial degradation.

N deposition effects on “new” (experimental-derived) biomarkers were only apparent for fungal biomarkers, while “old” (pre-experimental) biomarkers were often affected. Under increased N deposition, a retarded decomposition of “old” OM in fine mineral soil fractions was observed for both, plant and microbial biomarkers. These results support previous observations for total OM. The retarded decomposition of “old” OM might be attributed to reduced mining of microorganisms for N in native soil OM, if additional inorganic N is available. This mechanism might be especially important in fine mineral soil fractions, where OM is protected from microbial degradation by association with soil minerals.

Temperate forests are the major ecosystem in Europe and the reduced mining of microorganisms for native soil organic C under increased N deposition might affect the C balance of European soils, because it potentially increases soil C sequestration. However, on a global basis, temperate forest soils account for only 11 % of global soil C. In this respect, the study of tropical forest ecosystems is a potential area of future research with high relevance to global soil C storage.

ZUSAMMENFASSUNG

Anthropogene Tätigkeiten verändern durch steigende Emissionen von Kohlenstoffdioxid (CO_2) und reaktivem Stickstoff in die Atmosphäre die globalen biogeochemischen Kreisläufe von Kohlenstoff und Stickstoff. Ausschlaggebende Quellen sind die Verbrennung fossiler Brennstoffe sowie die Erzeugung und Ausbringung von Düngemitteln. Böden speichern global die grössten Mengen von terrestrischem Kohlenstoff und tauschen jährlich hohe Mengen an Kohlenstoff mit Pflanzen und der Atmosphäre aus. Unter den derzeitigen anthropogenen Umweltveränderungen ist jedoch nicht klar, ob Böden als Quellen oder Senken von Kohlenstoff wirken. Daher ist die Untersuchung der Auswirkungen von erhöhten atmosphärischen CO_2-Konzentrationen und Stickstoffdepositionen auf die Stabilisierung und den Umsatz der organischen Substanz in Pflanzen und Böden eine bedeutende Aufgabe.

Stabilisierungsmechanismen der organischen Bodensubstanz wurden jüngst mittels Dichtefraktionierung von Boden erforscht. Dies ermöglicht eine Trennung unterschiedlicher Bodenfraktionen, welche konzeptuell in Beziehung zu verschiedenen Stabilisierungsmechanismen stehen. Die Analyse stabiler Kohlenstoffisotope ($\delta^{13}C$) in Biomarkern bietet zudem die Möglichkeit, die Dynamik der organischen Substanz auf der molekularen Ebene zu verfolgen und zwischen organischer Substanz aus pflanzlichen und mikrobiellen Quellen zu unterscheiden. Die vorliegende Dissertation beabsichtigt, die Auswirkungen von erhöhten atmosphärischen CO_2-Konzentrationen und Stickstoffdepositionen auf die Stabilisierung und den Umsatz von pflanzlichen und mikrobiellen Biomarkern in Pflanzenbiomasse und Bodenfraktionen zu untersuchen.

Modellwaldökosysteme wurden mit Buchen und Fichten auf einem sauren Boden errichtet und mit umgebenden und erhöhten CO_2-Konzentrationen in Kombination mit zwei Stufen von Stickstoffdepositionen für vier Jahre behandelt. ^{13}C-abgereichertes CO_2 wurde benutzt, um zwischen „neuer“ (während dem Experiment entstandener) und „alter“ (vor dem Experiment bestehender) organischer Substanz zu unterscheiden. Stabile Kohlenstoffisotope ($\delta^{13}C$) von pflanzlichen (langkettige Fettsäuren) und mikrobiellen (Aminozucker) Biomarkern wurden in ober- und unterirdischer Biomasse der zwei Baumarten sowie in Bodendichtefraktionen analysiert.

Der gesamte organische Kohlenstoff und die mikrobiellen Biomarker (bakterielle und pilzliche) wurden hauptsächlich durch Assoziation mit Bodenmineralen stabilisiert. Bakterielle Biomarker waren an Bodenmineralen relativ angereichert im Vergleich zu pilzlichen Biomarkern. Im Gegensatz dazu akkumulierten pflanzliche Biomarker nicht an

Bodenmineralen, was darauf hinweist, dass diese nicht so effektiv durch Assoziation mit Bodenmineralen stabilisiert werden als der gesamte organische Kohlenstoff oder mikrobielle Biomarker. Diese Ergebnisse deuten darauf hin, dass die stabile organische Bodensubstanz hauptsächlich mikrobiellen Ursprungs und präferenziell an Bodenmineralen stabilisiert ist.

Pflanzliche und pilzliche Biomarker zeigten einen ähnlichen Umsatz im Vergleich zum gesamten organischen Kohlenstoff, während bakterielle Biomarker einen etwas niedrigeren Umsatz hatten. Die Ergebnisse deuten darauf hin, dass organische Bodensubstanz aus verschiedenen Quellen generell einen ähnlichen Umsatz hat und dass der Umsatz unabhängig von der chemischen Zusammensetzung ist. Der Umsatz der organischen Bodensubstanz scheint vielmehr von Prozessen wie Aggregation und Interaktion mit Bodenmineralen kontrolliert zu werden, welche die organische Bodensubstanz vor mikrobiellem Abbau schützen.

Auswirkungen von Stickstoffdeposition auf „neue“ Biomarker wurden nur für pilzliche Biomarker beobachtet, wohingegen „alte“ Biomarker öfter von Stickstoffdeposition beeinflusst wurden. Ein gehemmter Abbau von „alter“ organischer Substanz in feinen mineralischen Bodenfraktionen wurde für beide, pflanzliche sowie mikrobielle Biomarker unter erhöhter Stickstoffdeposition beobachtet. Diese Resultate bestätigen frühere Beobachtungen an der gesamten organischen Bodensubstanz. Der gehemmte Abbau „alter“ organischer Bodensubstanz kann auf ein vermindertes Ausbeuten der nativen organischen Bodensubstanz zur Stickstoffgewinnung durch Mikroorganismen zurückgeführt werden, wenn diese zusätzlich anorganischen Stickstoff zur Verfügung haben. Dieser Mechanismus scheint in feinen mineralischen Bodenfraktionen besonders wichtig zu sein, da die organische Bodensubstanz dort durch Assoziation mit Bodenmineralen vom mikrobiellen Abbau geschützt ist.

Temperierte Wälder sind die bedeutendsten Ökosysteme in Europa. Der gehemmte Abbau der nativen organischen Bodensubstanz durch Mikroorganismen unter erhöhten Stickstoffdepositionen könnte Auswirkungen auf die Kohlenstoffbilanz europäischer Böden haben, da es potentiell zu einer erhöhten Speicherung von Kohlenstoff in Böden kommen kann. Weltweit tragen Böden in temperierten Wäldern jedoch nur 11 % zum globalen Bodenkohlenstoff bei. Daher ist die Untersuchung tropischer Waldökosysteme ein potentielles Feld zukünftiger Forschung, da diese eine grössere Relevanz für die Speicherung von organischem Bodenkohlenstoff auf globaler Ebene haben.

CHAPTER 1

SYNOPSIS

This dissertation is a cumulative dissertation that is based on three individual studies. In this chapter, the main findings of the individual studies are summarized and an outlook for future research is given. Further results and a more detailed discussion can be found in the following chapters presenting the individual studies that are published in international peer-reviewed journals.

Introduction

The terrestrial carbon cycle in a changing environment

Humans are globally changing the environment by increasing atmospheric carbon dioxide (CO_2) concentrations and reactive nitrogen (N) deposition, which ultimately feeds back on global biogeochemical cycles and the climate system (Ciais *et al.*, 2013). Recently atmospheric CO_2 concentrations reached a landmark of 400 ppm and are exponentially increasing (Bala, 2013). Elevated CO_2 concentrations are expected to cause global warming with predicted increases of extreme climate events like droughts. Atmospheric N deposition increased three- to fivefold within the last century and is expected to further rise in the future (Figure 1; Ciais *et al.*, 2013). The main sources of reactive N in the atmosphere are combustion of fossil fuels and fertilizer application (Davidson, 2009). Reactive N is deposited from the atmosphere to terrestrial ecosystems by rainfall, which has a fertilizing effect on plant growth and microbial degradation. Ecosystems that generally do not receive fertilization, like forests, are most strongly affected by increased N deposition. In forests, N deposition affects key processes of the carbon (C) cycle, like photosynthesis or soil respiration, which has ecosystem-level implications (Zak *et al.*, 2011). Although there is a lot known about N effects on e.g. tree growth and litter decomposition, the effects of N deposition on the soil C cycle in forests are still under recent debate (Janssens *et al.*, 2010).

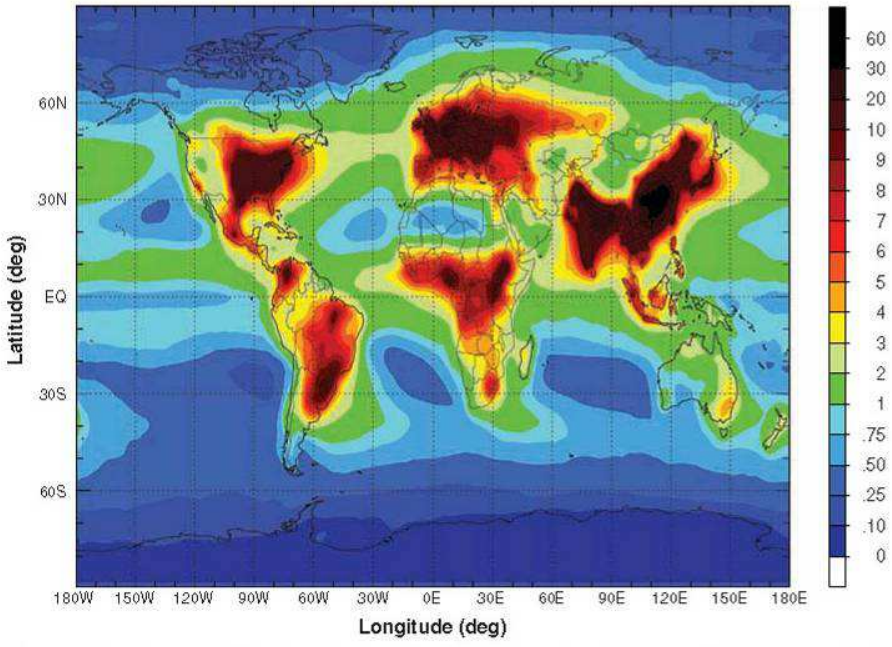

Figure 1 Estimated reactive nitrogen (N) deposition from global total N (NO_y and NH_x) emissions, totaling 105 Tg N yr^{-1}. The unit scale is kg N ha^{-1} vr^{-1}. Figure taken from Galloway *et al.* (2008).

Stabilization mechanisms of soil organic matter

Soils play a key role in the long-term storage of C in terrestrial ecosystems (Stockmann *et al.*, 2013). Globally, soils store about twice the amount of C that is stored in the atmosphere and four times the amount of C that is stored in all terrestrial biomass (Figure 2; Ciais *et al.*, 2013). Despite the large storage of C as soil organic matter (OM), soils also release large amounts of C as CO_2 to the atmosphere. Therefore, soils have a high potential for both storage and release of large C amounts. However, it is still not clear, if soils should be considered as sources or sinks of C under current climate change (Smith, 2012). Furthermore, the effects of increased N deposition on soil OM are not fully understood (Janssens *et al.*, 2010). Previous studies mainly concentrated on the sole analysis of total organic C in bulk soil, but the mechanisms behind stabilization and destabilization of OM in soils remain still unclear (Conant *et al.*, 2011; Schmidt *et al.*, 2011; Dungait *et al.*, 2012; Cotrufo *et al.*, 2013).

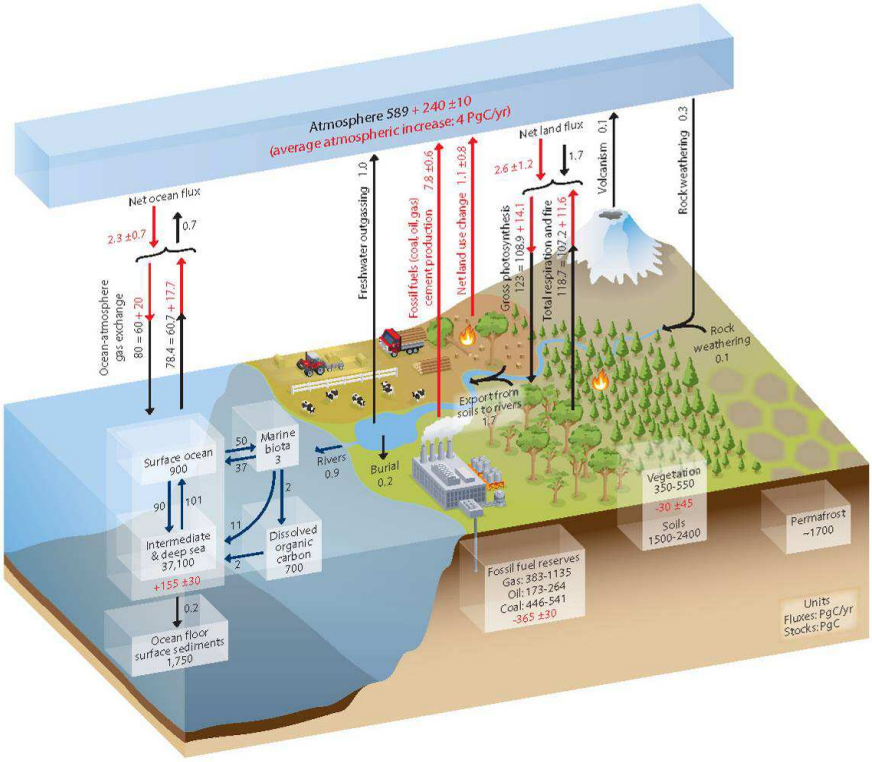

Figure 2 Simplified schematic of the global carbon (C) cycle. Numbers represent reservoir mass, also called 'carbon stocks' in PgC (1 PgC = 10^{15} gC) and annual carbon exchange fluxes (in PgC yr^{-1}). Black numbers and arrows indicate reservoir mass and exchange fluxes estimated for the time prior to the Industrial Era (circa 1750). Red arrows and numbers indicate annual 'anthropogenic' fluxes averaged over the 2000-2009 time period. These fluxes are a perturbation of the carbon cycle during Industrial Era post 1750. Red numbers in the reservoirs denote cumulative changes of anthropogenic carbon over the Industrial Era (1750-2011). Figure with modified caption taken from Ciais *et al.* (2013).

Biomarker and stable isotope analysis

In contrast to the study of bulk soil, physical soil fractionation can be used to investigate distinct soil fractions that should correspond to different stabilization mechanisms of soil OM (von Lützow *et al.*, 2007). The analysis of organic C content and isotopic composition in soil fractions is therefore of great value to elucidate the processes of soil OM stabilization. Furthermore, it is of high interest not only to investigate total organic C, but also the content and isotopic composition of specific biomarkers in soil fractions (Glaser, 2005; Amelung *et al.*, 2008). Biomarkers are organic compounds that have an organism-specific origin and are preserved in environmental archives like soils and sediments. Biomarkers can be used as proxies for source determination and taxonomic classification of plants and microorganisms. Plant- and microbial-derived biomarkers have been studied in agricultural, grassland and forest soils (Amelung *et al.*, 2008). However, most studies were applied in agricultural soils and they mainly analyzed bulk soil. Therefore, knowledge on the stabilization and turnover of biomarkers in forest soil fractions is missing. The combination of soil density fractionation with stable isotope biomarker analysis will provide novel insights into the dynamics of soil OM at the molecular scale (Schmidt *et al.*, 2011; Simpson & Simpson, 2012; Gleixner, 2013).

Objectives

The key objective of this dissertation is to understand how elevated atmospheric CO_2 concentrations and N deposition affect the dynamics of OM in forest soil density fractions. More specifically, the aim of this dissertation is to investigate biomarkers of specific origin to differentiate between microbial- and plant-derived OM in soil fractions. The dissertation uses a combination of several state-of-the-art techniques like soil density fractionation and isotope labeling in combination with stable C isotope analysis ($\delta^{13}C$) of biomarkers to answer the following questions:

Soil density fractionation methodology

Which values of methodological parameters (density and ultrasonic dispersion energy) are used in the literature? What are the reasons for the selection of parameter values? Are parameters values used consistently throughout different studies?

Stabilization and turnover of biomarkers in soil fractions

What are the quantities of plant- and microbial-derived biomarkers in forest soil density fractions and what is their stable C isotope composition ($\delta^{13}C$)? In which soil fractions are plant- and microbial-derived biomarkers stabilized? How is their turnover compared to that of total soil OM?

Climate change effects on biomarkers in soil fractions

How do elevated CO_2 concentrations and N deposition affect the dynamics of microbial- and plant-derived biomarkers in forest soil density fractions? Is there an interaction between the effects of elevated CO_2 concentrations and N deposition? Are there different effects of elevated CO_2 concentrations and N deposition on “new” (experimental-derived, < 4 years old) versus “old” (pre-experimental, > 4 years old) biomarkers in soil density fractions?

Experimental design

Model forest ecosystems

Archived plant and soil samples from a free air CO_2 enrichment (FACE) experiment that was conducted during four growing seasons between 1994 and 1998 were used in the present study. The experimental setup is described in detail in Egli *et al.* (1998). It consisted of model forest ecosystems established in open-top chambers with an acidic soil (pH = 4.1) with a sandy loamy texture, classified as Haplic Alisol that was planted with beech (*Fagus sylvatica*) and spruce (*Picea abies*) trees as well as five understory species (Figure 3).

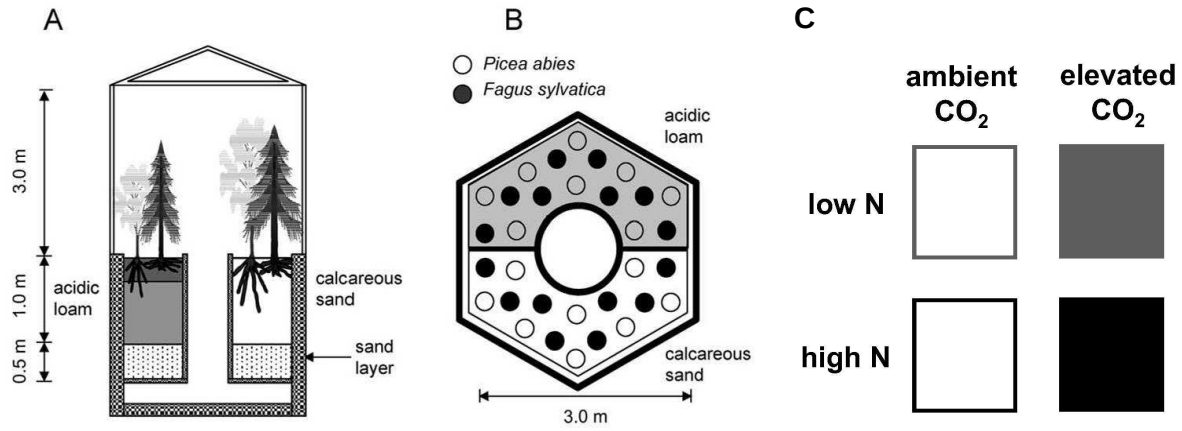

Figure 3 Model forest ecosystems. (A) Profile view of open-top chambers containing two lysimeters with different soil types. In this study only the acidic loam was used. (B) Planting scheme of spruce and beech trees. (C) Treatments. Figure was taken from Hagedorn *et al.* (2003).

Ecosystems were treated for four years with four different combinations of atmospheric CO_2 concentrations and N deposition and each treatment was replicated three times in the field. The treatments were as follows (see Figure 3C): ambient CO_2 (370 µmol mol^{-1}) + low N (7 kg NH_4NO_3-N) as a control; elevated CO_2 (570 µmol mol^{-1}) + low N; ambient CO_2 + high N (70 kg NH_4NO_3-N); elevated CO_2 + high N. The CO_2 in the elevated CO_2 treatments was depleted in ^{13}C compared to the ambient CO_2 treatment ($\Delta\delta^{13}C$ = 16 ‰) to isotopically label the ecosystems.

After four years of treatment above- and belowground plant biomass of the two species (beech leaves, beech roots, spruce needles, spruce roots) were sampled separately. Bulk soil was sampled from 0 to 10 cm depth. Prior to elemental and biomarker analyses bulk soil was separated into distinct soil fractions using a combination of density fractionation and ultrasonic dispersion.

Soil density fractionation

Soil fractionation is frequently used to separate bulk soil into distinct soil fractions that should ideally relate to different stabilization mechanisms of OM in soil (aggregation, association with soil minerals). In the present study, the density fractionation procedure of Golchin *et al.* (1994) was used to separate bulk soil into three density fractions (Figure 4).

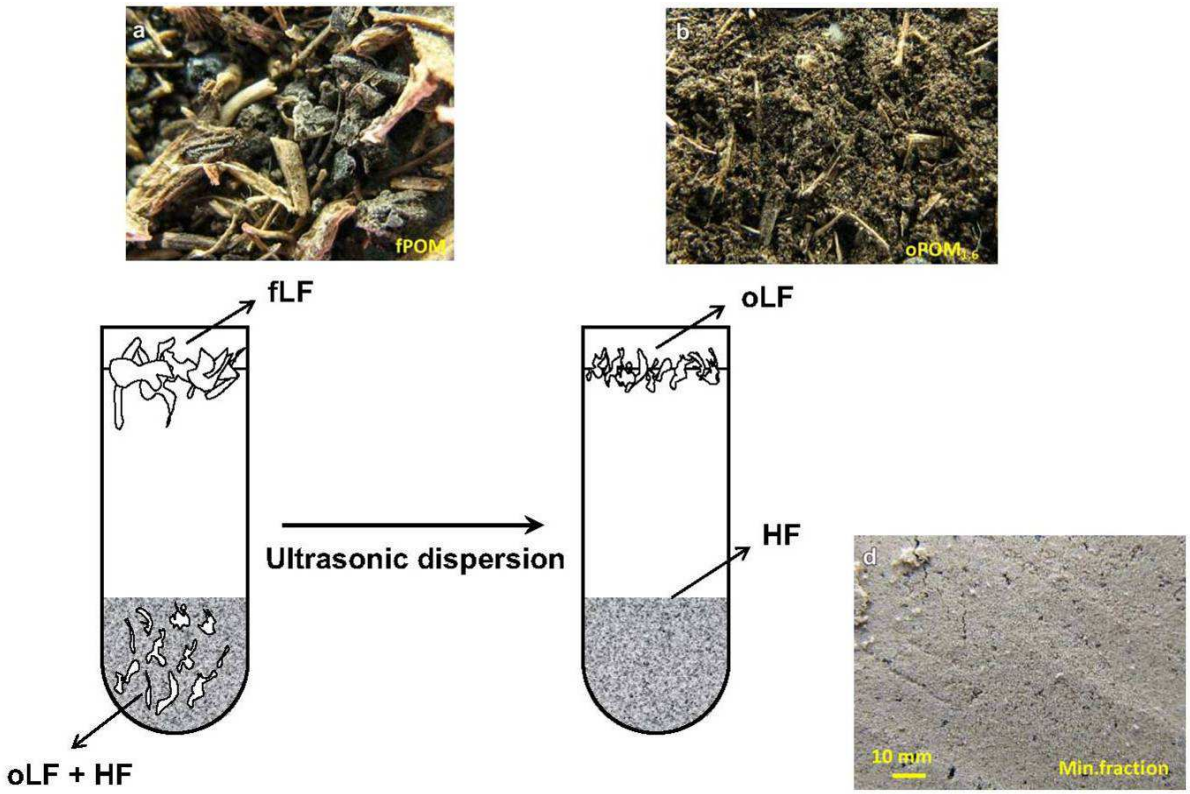

Figure 4 Schema of the methodological procedure for soil density fractionation according to Golchin *et al.* (1994). Density fractions are isolated by suspending soil in a solution of specific density and subsequent centrifugation. Free light fraction (fLF) material is floating while heavy fraction (HF) material sinks. Correspondingly, occluded light fraction (oLF) material is separated from HF after ultrasonic dispersion. Figure was redrawn after Wagai *et al.* (2009) and published in Griepentrog & Schmidt (2013). Photos were taken from Dorodnikov *et al.* (2011).

Bulk soil was separated into (a) the free light fraction representing recent, only partially degraded plant residues that are not physically protected from degradation, (b) occluded light fraction representing OM in soil aggregates and (c) heavy fraction representing OM associated with soil minerals. The heavy fraction was further separated by particle-size fractionation at 20 µm into a coarse (>20 µm) and a fine heavy fraction (<20 µm). The fractionation scheme used in the present study is shown in Figure 5.

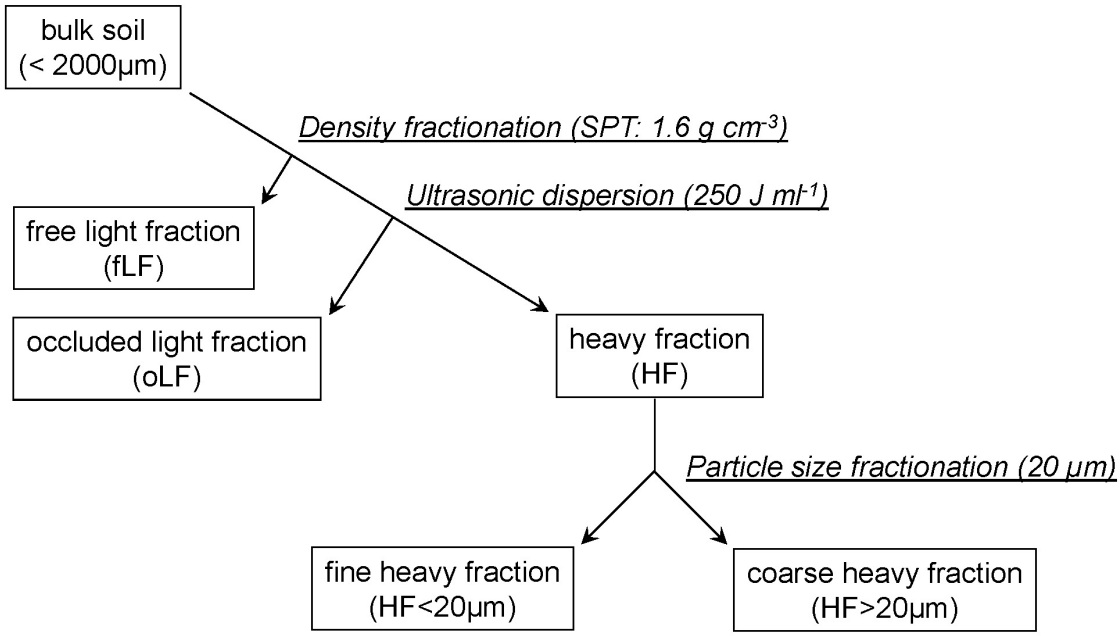

Figure 5 Soil fractionation scheme. Bulk soil was separated into free light fraction (fLF), occluded light fraction (oLF) and heavy fraction by density fractionation and ultrasonic dispersion. Heavy fraction was further particle-size fractionated at 20 µm into a coarse (HF > 20 µm) and a fine-textured heavy fraction (HF < 20 µm). Figure taken from Griepentrog *et al.* (2014a).

In order to determine the appropriate dispersion energy, bulk soil was sequentially fractionated with increasing dispersion energies (Cerli *et al.*, 2012). Results showed that 250 J ml^{-1} was the appropriate dispersion energy for our soil. A more detailed methodological description of the fractionation procedure can be found in Griepentrog *et al.* (2014a).

Total organic carbon and total nitrogen analysis

Total organic C and total N contents and stable C isotope ratios ($\delta^{13}C$) of plant biomass, bulk soil and soil fractions were determined with an automated element analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS). The results of the stable C isotope analysis are expressed in δ units (‰):

$$\delta^{13}C\,(‰) = 1000 \cdot \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \qquad (1)$$

where R = ^{13}C / ^{12}C for both, sample and standard. The Vienna Pee Dee Belemnite (VPDB) standard was used as reference.

Amino sugar analysis

Amino sugars were extracted from bulk soil and soil density fractions with acid hydrolysis using an adaption of the method described by Zhang & Amelung (1996). Compound-specific stable isotope analysis of amino sugar extracts was performed by using liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS) according to the method described by Bodé *et al.* (2009). A more detailed methodological description of amino sugar extraction and analysis can be found in Griepentrog *et al.* (2014a).

Bacterial- and fungal-derived amino sugar carbon (AS-C) was calculated according to Van Groenigen *et al.* (2007). Based on the assumption that all galactosamine and muramic acid are produced by bacteria and that glucosamine and muramic acid occur in equal amounts in bacteria

$$\text{Bacterial AS-C} = \text{galactosamine C} + 2 \cdot \text{muramic acid C} \quad (2)$$

The remainder of the amino sugar carbon pool is derived from fungi and therefore

$$\text{Fungal AS-C} = \text{glucosamine C} - \text{muramic acid C} \quad (3)$$

Fatty acids analysis

Total lipids were extracted from plant biomass, bulk soil and soil density fractions with a mixture of dichloromethane / methanol (9 / 1; v / v) using microwave extraction (Ernst *et al.*, 2013). Total lipid extracts were separated into neutral and fatty acid fractions via solid phase extraction using silica gel with 5% potassium hydroxide and sequential elution with solvents of different polarity. The fatty acid fraction was derivatized using boron trifluoride / methanol solution (BF_3 / MeOH). Prior to derivatization, deuteriated eicosanoic acid ($C_{20}D_{39}$) was added as an internal standard for quantification.

Compound identification and quantification was performed using a gas chromatograph coupled to a mass spectrometry detector (GC-MS). Compound-specific stable isotope analysis was performed using a gas chromatograph coupled to an isotope ratio mass spectrometer (GC-IRMS). A more detailed methodological description of fatty acid extraction and analysis can be found in Chapter 4 (Griepentrog *et al.*, 2014b).

Estimation of new versus old organic matter

Ecosystems were continuously treated with ^{13}C-depleted CO_2 to isotopically label plant biomass and estimate the fraction of C that was newly synthesized during the experimental period. The isotopic label could then be traced in total organic C and specific organic compounds (amino sugars or fatty acids) in above- and belowground plant biomass as well as bulk soil and distinct soil density fractions. Fraction of "new" soil C that is derived from plant biomass input during the experimental period was calculated from $\delta^{13}C$ values using a simple mixing model (Balesdent *et al.*, 1988):

$$F_{new} = \frac{\delta_{soil,depleted} - \delta_{soil,ambient}}{\delta_{plant,depleted} - \delta_{plant,ambient}} \quad (4)$$

where $\delta_{soil,depleted}$ and $\delta_{soil,ambient}$ are the $\delta^{13}C$ values of total organic C or specific compounds in bulk soil or soil fractions for treatments with ^{13}C-depleted CO_2 and ambient CO_2, respectively. Corresponding, $\delta_{plant,depleted}$ and $\delta_{plant,ambient}$ are the $\delta^{13}C$ values of total organic C or specific compounds in plant biomass for treatments with ^{13}C-depleted CO_2 and ambient CO_2, respectively.

Statistical analysis

The mean is given along with the standard error for replicate measurements (Webster, 2001). Means and standard errors are based on three field replicates if not indicated otherwise. Two-way analysis of variance was used to test the significance of treatment effects (elevated CO_2, N deposition) and their interactions (Webster, 2007).

Results and discussion

Soil fractionation methodology

A key part of this dissertation was the soil fractionation, preceding the (isotope) analyses of total organic C and distinct biomarkers for plant (long-chain fatty acids) and microbial (amino sugars) origin. Density fractionation of bulk soil is performed to gain distinct soil fractions that relate to different stabilization mechanisms of soil OM (Golchin *et al.*, 1994; Christensen, 2001; von Lützow *et al.*, 2007; Crow *et al.*, 2007; Wagai *et al.*, 2009). However, a recent study showed that it is crucial to systematically determine the appropriate values of methodological parameters (density and dispersion energy) of the density fractionation procedure each time for different soils (Cerli *et al.*, 2012). Nevertheless, looking into the literature, various different combinations of density and dispersion energy are used, but the criteria for their selection are obviously not transparent (Griepentrog & Schmidt, 2013).

Therefore, a literature review was conducted and methodological parameters from recent studies that use density fractionation along with ultrasonic dispersion were compiled. Results showed that there are striking discrepancies in the application of density fractionation methodology (Griepentrog & Schmidt, 2013). Apparently, there is no consensus on which parameter values to use and the reasons for the selection of fractionation parameters are rarely explained. Thus, it is not known how results of different approaches relate to each other and it is almost impossible to compare results of different studies. It is therefore recommended to test and report effects of fractionation parameter values on chemical and physical properties of soil fractions, to achieve agreement and coherence on parameters to be used and facilitate comparability in future studies (Griepentrog & Schmidt, 2013).

Bulk elemental composition of plant biomass and soil fractions

As a first step after the soil fractionation, total organic C and total N contents of plant biomass and soil fractions were determined. C contents were similar among all plant tissues, but due to different N concentrations, spruce needles had higher C/N ratios than beech leaves, while spruce roots shower lower C/N ratios than beech roots. Four years continuous treatment with ^{13}C-depleted CO_2 ($\Delta\delta^{13}C$ = 16 ‰) led to a significant decrease of $\delta^{13}C$ values of all plant tissues by circa 10 ‰ and an identical incorporation of isotope label was observed for different plant tissues. These results are in line with other free air CO_2 enrichment experiments (e.g. Van Kessel *et al.*, 2000).

In soil fractions, highest C concentrations were found in both light fractions, while heavy fractions had substantially lower C concentrations. Here fine heavy fractions showed higher C concentrations than coarse heavy fractions. C/N ratios were highest in both light fractions, decreased in coarse heavy fraction and were lowest in fine heavy fractions. These results are in line with the concept of the density fractionation approach (Golchin *et al.*, 1994; Cerli *et al.*, 2012). High C/N ratios in light fractions point towards large contribution of fresh OM originating from plant residues, while low C/N ratios in heavy fractions are attributed to increasing degree of OM degradation and higher contribution of microbial-derived OM.

Both light fractions together stored 29% of the total soil organic C, whereof around three quarters were stored in free light fractions and one quarter in occluded light fractions. The largest part of soil organic C was stored in fine heavy fractions (62%), whereas coarse heavy fraction contributed only 9% to total soil organic C. The high storage of C in fine heavy fractions can be attributed to enhanced protection of OM by association with soil minerals and interactions with clay minerals and Fe and Al oxides are thought to be most relevant for OM stabilization in soil (Kögel-Knabner *et al.*, 2008).

Soil fractions had significantly different C isotope compositions and ^{13}C abundance increased in the order: free light fraction < occluded light fraction < coarse heavy fraction < fine heavy fraction. Enrichment of ^{13}C is generally attributed to isotope fractionation during microbial degradation (Galimov, 2006) or preferential substrate utilization by microorganisms (Blagodatskaya *et al.*, 2011), but also to gradual shifts in the relative contribution of plant- versus microbial-derived compounds (Ehleringer *et al.*, 2000).

Four years of continuous treatments with ^{13}C-depleted CO_2 significantly decreased $\delta^{13}C$ values compared to treatment with ambient CO_2 for bulk soil and individual soil fractions. The difference between the treatments with ambient and ^{13}C-depleted CO_2 ($\Delta\delta^{13}C$) is a measure for the incorporation of isotopic label and hence for the fraction of “new” (experimental-derived) C. Percentage of “new” C decreased substantially from free light (60 %) to occluded light (21 %) fractions, showing that free light fractions mainly consist of recent plant-derived C and that the transfer of OM from free to occluded light fractions and the replacement of the OM pool in occluded light fractions is rather slow. Coarse heavy fraction showed similar percentage of “new” C compared with occluded light fractions, while fine heavy fractions had lowest percentage of “new” C (10 %). Here, the slow replacement of the OM pool is attributed to the large C pool in this fraction and furthermore to the stabilization of OM by interaction with fine soil minerals.

Biomarkers in plant biomass and soil fractions

In this dissertation, two different biomarkers, amino sugars and fatty acids, were analyzed for their molecular and stable C isotope composition ($\delta^{13}C$) in above- and belowground biomass of two tree species and in bulk soil and soil density fractions. Amino sugars were used as biomarkers for microbial-derived OM (bacterial versus fungal), while long-chain fatty acids ($>C_{20}$) were used as biomarkers for plant-derived OM. Short-chain fatty acids ($<C_{20}$) were also analyzed, but they cannot be attributed to a specific group of organisms, since they are produced by both, plants and microorganisms. It was investigated in which soil fraction plant- and microbial-derived biomarkers are stabilized and how their turnover is compared to that of total organic C.

Amino sugars

Concentrations of microbial biomarkers increased from free light to fine heavy fractions, showing that they are mainly associated with fine textured soil minerals. Furthermore, fungal-to-bacterial ratios decreased from free light to fine heavy fractions, showing that bacterial biomarkers are relatively enriched at mineral surfaces compared to fungal biomarkers. Potential reasons for the preferential association of bacterial biomarkers with soil minerals could be that in contrast to bacteria, most fungi are obligatory aerobes and are restricted to air-filled spaces in soil. Fungal hyphae were found to dominate in the outer regions of macroaggregates, whereas bacteria dominate in the center of microaggregates (Chenu & Stotzky, 2002).

In bulk soil and among all soil fractions, microbial biomarkers were enriched in ^{13}C compared to total organic C at natural abundance. Differences between $\delta^{13}C$ values of total organic C and microbial biomarkers results from isotope fractionation during biochemical synthesis and from different isotope signatures of the substrates used for biosynthesis (Hobbie *et al.*, 1999). Among soil fractions, $\delta^{13}C$ values of microbial biomarkers showed the same pattern than total organic C. Microbial biomarkers in heavy fractions showed higher $\delta^{13}C$ values than microbial biomarkers in light fractions. ^{13}C enrichment of microbial biomarkers in heavy fractions could be attributed to isotope fractionation during decomposition of microbial biomarkers (Galimov, 2006) or that the C source for microbial biomarker formation is taken from the ^{13}C-enriched organic C already present in heavy fractions.

Adding ^{13}C-depleted CO_2 for four years significantly decreased $\delta^{13}C$ values of microbial biomarkers compared to treatments with ambient CO_2. The percentage of “new” (experimental-derived) microbial biomarkers followed the pattern of total organic C, with the

highest percentage of “new” microbial biomarkers in free light fractions and considerably lower percentage of “new” microbial biomarkers in occluded light and heavy soil fractions. This shows that the replacement of the microbial biomarker pool in these fractions is slower than in free light fractions and it might be attributed to the stabilization of microbial biomarkers by aggregation in occluded light fractions or association with soil minerals in fine heavy fractions. However, also the pool size of microbial biomarkers is larger in heavy fractions compared with light fractions and therefore it takes longer until this pool replaced.

After four years of experimental period, the fraction of “new” fungal biomarkers was in the range of that of total organic C and higher than that of bacterial biomarkers throughout all soil fractions and bulk soil. This indicates that more fungal biomarkers were formed during the experimental period and that fungal biomarkers have a slightly higher turnover compared to bacterial biomarkers.

Fatty acids

Fatty acids showed distinct distribution patterns between different plant species and between above- versus belowground plant biomass, while distribution patterns were similar among different soil fractions and bulk soil. The comparison of distribution patterns of fatty acids in plant biomass and soil fractions suggest a high contribution of root-derived fatty acids to soil OM. Concentrations of fatty acids decreased from free light to fine heavy fractions, which is in contrast to the observations made for amino sugars. This suggests that fatty acids are not as effectively stabilized by association with soil minerals than microbial sugars or total organic C, which might be related to the fast incorporation of fatty acids in soils (Wiesenberg *et al.*, 2010).

Fractions of “new” (experimental-derived) fatty acids decreased from free light to fine heavy fractions. After four years of experimental period, the fraction of “new” long-chain fatty acids equaled that of total organic C. Short-chain fatty acids had significantly higher fractions of “new” C compared to long-chain fatty acids and total organic C throughout all soil fractions and bulk soil. This indicates that more short-chain fatty acids were formed during the experimental period and that they have a higher turnover compared to long-chain fatty acids and total organic C. This might be explained by the additional contribution of microorganisms to short-chain fatty acids, which generally show a faster incorporation of isotope label compared to plant biomass (Kramer & Gleixner, 2006). However, also a fast incorporation of plant-derived short-chain fatty acids, which are partially contained in root exudates, could potentially contribute to the observations (Wiesenberg *et al.*, 2010).

Effects of nitrogen deposition and CO_2 concentrations

This dissertation investigated the interactive effects of elevated atmospheric CO_2 concentrations and increased N deposition on the dynamics of total organic C as well as plant- and microbial-derived biomarkers in plant biomass and soil fractions. Furthermore, it was tested, if elevated atmospheric CO_2 concentrations and increased N deposition differently affect "new" (experimental-derived) OM in contrast to "old" (pre-experimental) OM.

Bulk elemental composition

Spruce needles were significantly affected by treatments with elevated atmospheric CO_2 concentrations and increased N deposition. Under elevated CO_2, N concentrations decreased and C/N ratios increased, while the opposite was true under increased N deposition, which increased N concentrations and decreased C/N ratios. Contrasting effects of elevated CO_2 and increased N deposition on the chemical composition of plant biomass have been previously observed (Saxe *et al.*, 1998; Hyvönen *et al.*, 2007) and are attributed to changes in photosynthesis (Ainsworth & Long, 2005) or OM allocation within the plant (Dieleman *et al.*, 2010).

Increased N deposition furthermore significantly decreased C/N ratios in light fractions. These effects are in line with effects observed for plant biomass and plausible since light fractions mainly consist of recent, partially degraded plant biomass.

Increased N deposition did not significantly affect the C isotope ratios ($\delta^{13}C$) of total organic C in plant biomass, soil fractions and bulk soil. However, increased N deposition significantly increased the amount of "new" (experimental-derived) C in coarse heavy fractions, but due to the small amounts of C that is stored in coarse heavy fractions, this increase might not be relevant for total soil C sequestration.

Amino sugars

Microbial biomarkers were not affected by elevated atmospheric CO_2 concentrations, but increased N deposition affected both, "new" (experimental-derived) and "old" (pre-experimental) microbial biomarkers in soil fractions and bulk soil. High N deposition increased the amount of "new" fungal biomarkers in bulk soil. Hence, fungal biomass increased during the experiment, which is also supported by increased ergosterol concentrations in the same experiment (Wiemken *et al.*, 2001a). The likely reason for the increase in "new" fungal biomarkers under high N deposition is the N-induced growth

stimulation found in this experiment, which significantly increased fine root biomass and associated ectomycorrhizal fungi (Wiemken *et al.*, 2001b).

Furthermore, higher amounts of "old" microbial biomarkers in fine mineral fractions were found under high compared to low N deposition, showing that decomposition of "old" microbial biomarkers was retarded under increased N deposition. A possible explanation for the retarded decomposition of "old" microbial biomarkers could be reduced mining of native OM by microorganisms, if additional inorganic N is available (Fontaine *et al.*, 2011). This mechanism might be especially important in fine mineral fractions where OM is effectively protected from decomposition by association with soil minerals (Griepentrog *et al.*, 2014a).

Fatty acids

In plant biomass, short-chain and long-chain fatty acids were both affected by increased N deposition, which leads to higher $\delta^{13}C$ values. However, N effects were only apparent under elevated CO_2, showing a significant interaction between N deposition and CO_2 concentrations (except for spruce roots). The interactive effects of increased N deposition and elevated CO_2 are likely the result of changes in C isotope fractionation during photosynthesis, as it was previously found for birch seedlings by Huang *et al.* (1999). At elevated atmospheric CO_2 concentrations the ratio of partial pressure between the CO_2 concentrations inside and outside the leaves changes, which results in closure of stomata (Ainsworth & Rogers, 2007). Simultaneously, N deposition stimulates plant growth and promotes photosynthesis, which increases the consumption of intercellular CO_2 and reduces intercellular partial pressure (Huang *et al.*, 1999). The combined effects of reduction in stomatal conductance and increase in intercellular CO_2 consumption lead to a reduction of ^{13}C discrimination during photosynthesis and consequently to higher $\delta^{13}C$ values of plant biomass (Farquhar *et al.*, 1989).

In bulk soil and soil fractions, short-chain fatty acids showed the same significant N effects on $\delta^{13}C$ values as observed for plant biomass (except for fine mineral fractions). In contrast, long-chain fatty acids in bulk soil and soil fractions were not significantly affected by N deposition. N effects in soil fractions might be attributed to the observed changes in $\delta^{13}C$ values of plant biomass input, but also to N effects on microbial-derived short-chain fatty acids. The latter might be more likely, since no effects could be observed on plant-derived long-chain fatty acids in bulk soil and soil fractions. This suggests that N deposition effects in soil fractions are mainly attributed to microorganisms and only in minor parts to changes in composition of plant-derived OM.

Although increased N deposition affected the C isotope composition ($\delta^{13}C$) of fatty acids, N deposition did not significantly affects the percentage and total amounts of “new” fatty acid C in soil fractions and bulk soil. The absence of significant effects might be attributed to large errors associated with the analysis and calculation of “new” C fractions, but also to the relatively short duration of the experiment of only four years.

In contrast to “new” fatty acid C, the amounts of “old” (pre-experimental) long-chain fatty acid C were 7.8 % higher in fine mineral fractions and 15.4 % higher in bulk soil under increased N deposition compared to control treatments. However, the effects were not of high statistical significance ($p = 0.094$ for fine mineral fractions; $p = 0.075$ for bulk soil). The weak statistical significance of the results might again be attributed to the short duration of the experiment, the large soil C stocks and high uncertainty in calculating amounts of “new” and “old” C. However, higher amounts of “old” fatty acid C under increased N deposition are in agreement with other findings from the same experiment that show a retarded decomposition of “old” total organic C (Hagedorn *et al.*, 2003) and “old” fungal biomarker C (Griepentrog *et al.*, 2014a) under increased N deposition. The decrease in decomposition of “old” C under high N deposition could be attributed to reduced mining of native soil OM by microorganisms, if additional inorganic N is available (Fontaine *et al.*, 2011). This process seems to be especially important in soil fractions where OM is effectively protected from degradation by association with soil minerals.

Conclusions

The aim of this dissertation was to investigate OM dynamics in temperate forest ecosystems at the molecular level. Isotope labeling and stable C isotope analysis ($\delta^{13}C$) of microbial- and plant-derived biomarkers were used to trace biomarkers in above- and belowground plant biomass and soil density fractions. The effects of elevated atmospheric CO_2 concentrations and N deposition on the stabilization and turnover of these biomarkers in forest soil density fractions were studied. The following points represent highlights of this dissertation.

Soil fractionation methodology

A literature review revealed that there are discrepancies in the utilization of density fractionation methodology. Density cut-off and dispersion energy are crucial methodological parameters to yield fractions that correspond to their conceptual definitions (Cerli *et al.*, 2012). However, reasons for the selection of parameter values are rarely given and it is thus not known how results from different approaches relate to each other. It is recommended to test and report effects of parameter values on chemical and physical properties of soil fractions to achieve coherence in the use of these parameters. This would facilitate the comparability of future studies and allow systematic comparisons across different soil types.

Stabilization and turnover of biomarkers in soil fractions

Microbial (especially bacterial) biomarkers were mainly stabilized by association with soil minerals, which was also the case for total organic C in this experiment. In contrast, plant biomarkers were mainly found in physically unprotected soil fractions and their content was lowest in mineral soil fractions. This suggests that plant biomarkers are not as effectively stabilized by association with soil minerals as microbial biomarkers and total organic C. The results support recent studies that use isotope labeling in combination with direct observations at the submicron scale (Miltner *et al.*, 2012; Vogel *et al.*, 2014) and are a further indication that stable OM in soils is mainly of microbial origin and preferentially stabilized at soil minerals.

Plant and fungal biomarkers showed similar turnover compared to total organic C in soil fractions, while slightly lower turnover was found for bacterial biomarkers. However, differences were not large and it seems that the chemical structure of a compound is not a major factor that determines its turnover in soil. Instead, soil OM turnover seems to be

controlled by processes like aggregation and interaction with soil minerals that protect soil OM from microbial degradation.

Nitrogen deposition effects on biomarkers in soil fractions

N deposition had only minor effects on “new” (experimental-derived) biomarkers. However, the production of “new” fungal biomarkers increased in bulk soil by 108% under high N deposition. A retarded decomposition of “old” (pre-experimental) biomarkers in fine mineral soil fractions was observed for fungal biomarkers (+25 %) and plant biomarkers (+15 %) and supports previous observations for total soil OM (Hagedorn *et al.*, 2003). The retarded decomposition of “old” OM can be attributed to reduced mining of native soil OM by microorganisms, if they receive additional inorganic N. This mechanism seems to be especially important in fine mineral soil fractions, where OM is effectively protected from microbial degradation by association with soil minerals.

Temperate forests are the major ecosystem in Europe and account for 40 % of the land area (Schulze *et al.*, 2009). The reduced mining of native soil OM by microorganisms under increased N deposition might impact the C balance of European soils due to the potential increases of soil C sequestration. However, on a global scale, temperate forest soils cover only 9 % of the land area and store 11 % of global soil C (Schlesinger & Bernhardt, 2013). It is therefore of future interest to study ecosystems with larger relevance to global soil C storage (e.g. tropical forests).

Research perspectives

In this dissertation, stable C isotope analysis ($\delta^{13}C$) of biomarkers was applied to study the effects of elevated atmospheric CO_2 concentrations and N deposition on OM dynamics in soil fractions at the molecular level. In the following, a selection of possible lines for future research is presented.

Underrepresented ecosystems with large soil carbon stocks

Under current environmental change, soils may act as sources or sinks of C and it is therefore of special concern to investigate ecosystems that are underrepresented in current studies, but of high relevance to the storage of global soil C (2300 Pg C; Schlesinger & Bernhardt, 2013). Ecosystems that store the largest amounts of C in their soils are tropical forests (30 % of global soil C; Schlesinger & Bernhardt, 2013) and permafrost regions (which account for additional 1700 Pg C; Ciais *et al.*, 2013).

Studies at larger temporal and spatial scales

The present study was conducted on relatively small spatial and temporal scales (young trees in open-top chambers for 4 years). It is of future interest to conduct studies on larger spatial and / or temporal scales (mature forests in the field for several decades), to close existing knowledge gaps at different scales (Gärdenäs *et al.*, 2011). Today, there are only a very small number of long-term experiments that manipulate both atmospheric CO_2 concentrations and N deposition and from which it is possible to extrapolate to the globe (Reich *et al.*, 2006).

Multiple factor interactions

Apart from elevated atmospheric CO_2 concentrations and N deposition, also increases in temperature and changes of precipitation patterns are expected due to climate change in the future (Ciais *et al.*, 2013). However, the temperature sensitivity on soil OM dynamics is still not resolved, due to contradictory results of laboratory and field experiments (Conant *et al.*, 2011). Therefore, further research on the interactive effects of multiple environmental factors (temperature, moisture, CO_2, N) is needed to understand the processes that control soil OM dynamics under the current environmental change.

Dynamics of other biomarkers in soil fractions

Further research is also required to study how other significant biomarkers are distributed and stabilized in soil density fractions, to get deeper insights into soil OM dynamics at the

molecular level. Other recently investigated biomarkers of potential interest are e.g. cutin and suberin to study above- versus belowground plant inputs (Andreetta *et al.*, 2013), which is of interest because recent evidence has shown that soil OM is in large parts derived from roots (Schmidt *et al.*, 2011). Also of recent interest are glycerol dialkyl glycerol tetraether (GDGT) lipids of microbial origin, which are increasingly used as a proxy for temperature and pH in soils (Schouten *et al.*, 2013).

Linking carbon and nitrogen dynamics at the molecular level

Apart from analyzing other biomarkers for their C isotopic composition ($\delta^{13}C$) it would be of even higher relevance to additionally analyze N containing biomarkers (e.g. amino sugars) for their N isotope composition ($\delta^{15}N$) using compound-specific stable isotope analysis (Walsh *et al.*, 2014). Combining isotope labeling with both $\delta^{13}C$ and $\delta^{15}N$ analysis of specific biomarkers would allow the simultaneous tracing of C and N fluxes at the molecular level.

Environmental constraints on plant hydrological studies

Hydrogen isotopes ($\delta^{2}H$) of plant waxes (*n*-alkanes) are emerging as proxies for plant hydrological conditions over different temporal and spatial scales (Sachse *et al.*, 2012). However, apart from hydrological conditions (changing precipitation patterns), also other environmental factors like N deposition and elevated CO_2 might affect the hydrogen isotope composition of plant waxes. Thus, further insights would be important for interpretation of plant hydrology but might also result in novel environmental proxies.

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CHAPTER 2

SHORT COMMUNICATION

DISCREPANCIES IN THE UTILIZATION OF DENSITY FRACTIONATION ALONG WITH ULTRASONIC DISPERSION TO OBTAIN DISTINCT POOLS OF SOIL ORGANIC MATTER

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Author contributions

Marco Griepentrog wrote the manuscript under supervision of Michael W. I. Schmidt.

Abstract

Density fractionation along with ultrasonic dispersion is widely applied in soil science to obtain distinct fractions of soil organic matter. Density cut-off and dispersion energy are crucial parameters to yield fractions closely corresponding to their conceptual definitions. Our literature review revealed methodological discrepancies in the utilization of density fractionation schemes. Strikingly, reasons for the selection of specific density or dispersion energy were rarely explained. Thus, it is not known how results of different approaches relate to each other. We therefore recommend testing and reporting effects of fractionation parameters on chemical and physical properties of fractions, to achieve agreement and coherence on parameters to be used and facilitate comparability in future studies.

Introduction

In soil science, density fractionation has been used since the 1960s to separate soil organic matter (SOM) into discrete fractions that conceptually represent different stages of degradation and association with minerals (*Christensen*, 1992; *Sollins* et al., 1999). Basically these stages represent (1) the light fraction (LF) which is recent, partially decomposed SOM not firmly associated with soil minerals, and (2) the heavy fraction (HF) which is decomposed SOM, already associated with mineral surfaces (*Crow* et al., 2007). Methodologically these fractions are isolated by suspending soil in a solution of specific density (nowadays sodiumpolytungstate) and subsequent centrifugation, which leaves the LF material floating while HF material sinks (Figure 1). This basic two-fractions scheme was extended by conceptually dividing the light fraction into an inter-aggregate, "free" light fraction (fLF) and an intra-aggregate, "occluded" light fraction (oLF) (*Golchin* et al., 1994). Conceptually, the oLF represents further decomposed light fraction material, which is already occluded within soil aggregates, but not yet strongly associated with soil minerals. Methodologically, fLF material equals LF material in the two-fractions scheme and is separated only by floatation without ultrasonic dispersion, whereas oLF material is separated by floatation after ultrasonic dispersion of the resuspended HF material (Figure 1). This three-fractions scheme has been applied in numerous studies by combining density fractionation with ultrasonic dispersion (Table 1). Density cut-off and ultrasonic dispersion energy are the two most critical parameters to obtain SOM fractions closely corresponding to their conceptual definitions, because they affect quality and quantity of the fractions gained (*Kaiser* and *Guggenberger*, 2007; *Paré* and *Bedard-Haughn*, 2011; *Cerli* et al., 2012). However, the chemical composition of the occluded light fraction was found to be significantly more variable than that of free light and heavy fractions, and inconsistent with current conceptual models (*Wagai* et al., 2009). Possible explanations could be that processes underlying the formation of material found in the occluded light fraction material might be more complex than assumed (*Wagai* et al., 2009), or that the ultrasonic treatment had created methodological artifacts. In this short communication, we review how consistent density and ultrasonic dispersion energy are applied in a frequently used (three-pool) fractionation scheme (fLF, oLF, HF).

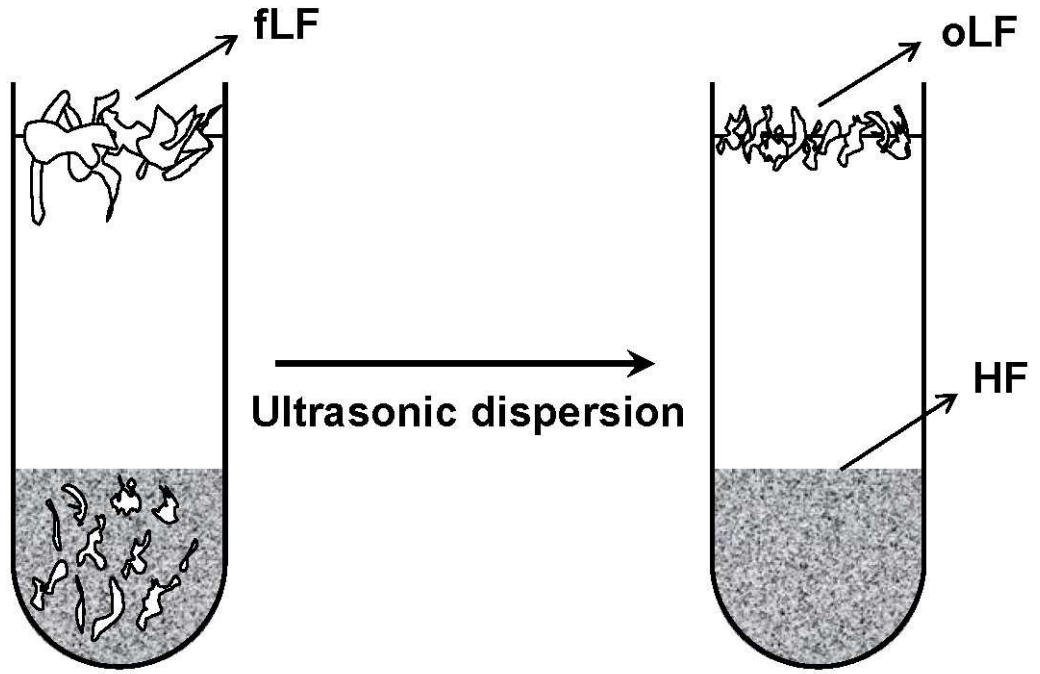

Figure 1 Schema of the methodological procedure for soil density fractionation according to *Golchin* et al. (1994). Density fractions are isolated by suspending soil in a solution of specific density and subsequent centrifugation. Free light fraction (fLF) material is floating while heavy fraction (HF) material sinks. Correspondingly, occluded light fraction (oLF) material is separated from HF after ultrasonic dispersion. Figure was redrawn after *Wagai* et al. (2009).

Materials and methods

From 35 published studies we extracted, the two most critical parameters affecting quality and quantity of soil fractions: (1) density cut-offs used to separate light from heavy fraction material, (2) ultrasonic dispersion energies used to separate the occluded light fraction within soil aggregates (Table 1). Density cut-offs were always reported as mass per volume (g cm^{-3}). Ultrasonic dispersion energies were commonly reported as energy per volume of suspension (J ml^{-1}), but other units (e.g. energy per mass of soil (J g^{-1})) were also frequently reported. If possible, we transformed values into J ml^{-1} for better comparison. Additionally, we recorded according to which method the ultrasonic equipment had been calibrated and if possible, the clay and bulk organic carbon content of the soil samples (Table 1). In this short communication we focus on studies that use a three fractions scheme (fLF, oLF, HF) according to *Golchin* et al. (1994). For reasons of conceptual and methodological comparability we excluded studies that use other separation schemes to isolate light and heavy fractions (e.g. sequential densities and combinations with particle-size or chemical fractionation).

Results and discussion

Densities to separate light and heavy fractions ranged from 1.6 to 1.9 g cm^{-3}, with most commonly used densities being 1.6 (n = 8) and 1.8 g cm^{-3} (n = 20). Most importantly, reasons for density selection that are based on experimental evidence are rarely reported. This seems surprising, since it could be shown, that variations in density cut-offs significantly affected the quality and quantity of the fractions gained (*Sollins* et al., 1999; *Crow* et al., 2007; *Kaiser* and *Guggenberger*, 2007; *Paré* and *Bedard-Haughn*, 2011; *Cerli* et al., 2012). *Sollins* et al. (1999) already stated that 1.6 to 1.7 g cm^{-3} is often a convenient cut-off to separate light and heavy fractions, but it might not be applicable to all soils. They recommended that each group of soils should be checked initially at a series of densities and the resulting fractions should be analyzed for ash content, C content, and C:N ratio, to select an optimal density cut-off for each soil. The density above which ash content of light fraction increases or C content decreases or at which the difference in C:N ratio of light and heavy fraction peaks, will be optimal for separating a "meaningful light fraction". However, the first study that did this exercise over a wide range of soils (n=16), was published only recently by *Cerli* et al. (2012), and they found that 1.6 g cm^{-3} works well for most soil types. This finding is in line with other studies that only investigated a smaller number of soils and found redistribution of mineral particles from heavy into light fractions at densities above 1.6 g cm^{-3}, where mineral particles would float and increase the mass of light fraction, but dilute its carbon content (*Crow* et al., 2007; *Kaiser* and *Guggenberger*, 2007; *Paré* and *Bedard-Haughn*, 2011).

Dispersion energies varied between 22 and 1800 J ml^{-1}, comprising almost two orders of magnitude. However, often dispersion energies were not reported, or it was not possible to convert the reported units into the commonly used unit J ml^{-1}, since essential information on soil mass and volume of density solution were missing. Interestingly, calibration of the ultrasonic equipment was only reported in 17 out of the 35 studies, although it is well known that the actual and nominal energy output can deviate (*Schmidt* et al., 1999). Thus, ultrasonic dispersion energies should be reported using calibrated ultrasonic equipment, along with the essential information (1) mass of soil, (2) volume of solution, (3) rate of energy input per volume of solution + soil, to facilitate comparability of dispersion energies in future studies.

Table 1 Studies that use combinations of density fractionation and ultrasonic dispersion to obtain distinct pools of soil organic matter (free light fraction, occluded light fraction, heavy fraction). References with information on density cut-off used to separate light from heavy fraction material, ultrasonic dispersion energy used to release occluded light fraction material, calibration method of ultrasonic equipment, as well as range of clay and bulk organic carbon content of the fractionated soil samples.

	Ultrasonic dispersion energy				
Density cut-off ($g\ cm^{-3}$)	Rate ($J\ ml^{-1}$)	Calibration method	Clay content (%)	Organic carbon content ($g\ kg^{-1}$)	Reference
1.6	60	b	—	2.4 - 70.8	*Grünewald et al.* (2006)
	200	b	2 - 6	2.3 - 39.2	*Cerli et al.* (2009)
	450	b	5.1 - 30.2	—	*Don et al.* (2009)
	450	c	—	9.1 - 35.9	*Wiedemeier et al.* (2012)
	656	a	9.5 - 45.6	29.8 - 142	*Wagai et al.* (2008)
	750*	—	12 - 72	5.5 - 8.0	*Golchin et al.* (1994)
	1800*	—	—	6.2 - 286	*Rovira* and *Vallejo* (2003)
	x	a	—	—	*Rasmussen et al.* (2005)
1.7	200	—	—	5.9 - 24.9	*Swanston et al.* (2005)
	225	—	—	—	*McLauchlan* and *Hobbie* (2004)
	270	c	84 - 90	12.9 - 64.7	*Roscoe et al.* (2001)
	270	c	84 - 87	32.3 - 64.7	*Roscoe et al.* (2004)
	800	c	—	27.3 - 41.8	*Roscoe & Buurman* (2003)
	x	—	8.7	—	*Parker et al.* (2002)
1.8	22	—	1 - 24	11 - 279	*Budge et al.* (2011)
	22	a	14 - 22	11.7 - 38.8	*Leifeld* and *Kögel-Knabner* (2005)
	200	—	—	—	*Marin-Spiotta* et al. (2008)
	200	—	25 - 36	—	*Marin-Spiotta* et al. (2009)
	300	a	—	15.6 - 65.1	*Llorente* et al. (2010a)
	300	a	—	15.6 - 65.1	*Llorente* et al. (2010b)
	400	—	56 - 63	17.7 - 45.0	*Freixo* et al. (2002a)
	400	—	48 - 58	11.9 - 46.8	*Freixo* et al. (2002b)
	400	—	19.3 - 36.0	6.8 - 18.5	*Macedo* et al. (2008)
	440	—	16.1 - 24.6	9.2 - 90.6	*Müller* and *Kögel-Knabner* (2009)
	450	b	6 - 30	12.0 - 20.3	*Kölbl* and *Kögel-Knabner* (2004)
	900*	—	—	9.3 - 21.5	*Wang* and *Wang* (2011)
	900*	—	25.4	—	*Wang* et al. (2005)
	x	—	—	4.0	*Fornara* et al. (2011)
	x	—	33.6	17.0	*Mubarak* and *Rosenani* (2003)
	x	a	—	—	*Murage* et al. (2007)
	x	—	20.0	—	*Poirier* et al. (2005)
	x	a	—	4.0 - 8.2	*Rasmussen* and *White* (2010)
	x	a	14 - 50	6.5 - 26.3	*Sohi* et al. (2001)
	x	a	18 - 66	—	*Sohi* et al. (2005)
1.9	x	—	—	7.4 - 54.4	*Basile-Doelsch* et al. (2009)

* value converted from other unit (e.g. $J\ g^{-1}$)
x value could not be converted
— not reported

a *North* (1976)
b *Schmidt* et al. (1999)
c *Roscoe* et al. (2000)

Even more importantly, the reason why a specific dispersion energy had been selected, rarely has been discussed. As an attempt to further explore if clay or carbon content were the selection criteria in the reviewed studies, we hypothesized that dispersion energies increase at higher contents of clay or organic carbon. However, we could not find any relationship (Table 1). Dispersion energies to disrupt aggregates need to be adjusted to the texture and carbon content of the sample material used. Excess dispersion energy would transfer minerals from heavy material to light fractions, with organic rich samples being more vulnerable to this transfer (*Kaiser* and *Guggenberger*, 2007). Different dispersion energies affected not only mass recovery, but also contents of carbon and lignin-derived phenols of the separated fractions (*Cerli* et al., 2012), again illustrating the importance to test the effects of fractionation parameters on the composition of individual density fractions. A simple and straightforward experiment could be to separate fractions with different amounts of dispersion energy and determining mass recovery and carbon contents of the light fractions (e.g. *Golchin* et al., 1994; *Sollins* et al., 1999; *Sohi* et al., 2001; *Cerli* et al., 2012). According to the underlying concept, light fraction material should have maximum organic carbon content to exclude contamination with minerals and organic material from heavy fractions.

Conclusion

Based on the review of published literature, we found it almost impossible to compare results of density fractionation procedures from different studies for two reasons. First, dispersion energies were reported in different units and calibrated or non-calibrated. For better comparability of future studies, we recommend to report dispersion energies along with mass of soil, volume of solution and energy rate per soil and solution using calibrated ultrasonic equipment. Second, it is difficult to find studies reporting the selection criteria for density cut-off and dispersion energy. We recommend to select dispersion energy based on experimental evidence (e.g. which energy produced the highest carbon yield of the light fraction) and to report the results. Following these simple recommendations would facilitate comparability of results from different studies, and allow systematic comparisons across soil types, which are desperately needed.

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CHAPTER 3

NITROGEN DEPOSITION PROMOTES THE PRODUCTION OF NEW FUNGAL RESIDUES BUT RETARDS THE DECOMPOSITION OF OLD RESIDUES IN FOREST SOIL FRACTIONS

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Author contributions

Frank Hagedorn, Alexander Heim and Michael W. I. Schmidt proposed the study. Pascal Boeckx and Samuel Bodé made the compound-specific stable-isotope-analysis of amino sugars possible and gave conceptual and technical support. Marco Griepentrog conducted the laboratory work, analyzed the data and wrote the manuscript. All authors contributed with constructive comments to the final version of the manuscript.

Abstract

Atmospheric nitrogen (N) deposition has frequently been observed to increase soil carbon (C) storage in forests, but the underlying mechanisms still remain unclear. Changes in microbial community composition and substrate use are hypothesized to be one of the key mechanisms affected by N inputs. Here, we investigated the effects of N deposition on amino sugars, which are used as biomarkers for fungal- and bacterial-derived microbial residues in soil. We made use of a four year combined CO_2 enrichment and N deposition experiment in model forest ecosystems, providing a distinct ^{13}C signal for "new" and "old" C in soil organic matter and microbial residues measured in density and particle-size fractions of soils. Our hypothesis was that N deposition decreases the amount of fungal residues in soils, with the new microbial residues being more strongly affected than old residues. The soil fractionation showed that organic matter and microbial residues are mainly stabilized by association with soil minerals in the heavy and fine fractions. Moreover, the bacterial residues are relatively enriched at mineral surfaces compared to fungal residues. The ^{13}C tracing indicated a greater formation of fungal residues compared to bacterial residues after four years of experiment. In contradiction to our hypotheses, N deposition significantly increased the amount of new fungal residues in bulk soil and decreased the decomposition of old microbial residues associated with soil minerals. The preservation of old microbial residues could be due to decreased N limitation of microorganisms and therefore a reduced dependence on organic N sources. This mechanism might be especially important in fine heavy fractions with low C/N ratios, where microbial residues are effectively protected from decomposition by association with soil minerals.

Introduction

Atmospheric nitrogen (N) deposition increased three- to fivefold within the last century due to human activities (Denman *et al.*, 2007). The main sources of reactive N in the atmosphere are combustion of fossil fuels and fertilizer application including animal manure (Davidson, 2009). Atmospheric N is mainly deposited to terrestrial ecosystems by rainfall in readily bioavailable forms, which has a fertilizing effect. Ecosystems that typically do not receive additional amounts of N and especially forests in industrialized regions are affected by increased N deposition in several ways. N deposition stimulates photosynthesis and enhances the allocation shift to more aboveground versus belowground plant growth, thereby reducing organic matter inputs into the rhizosphere (Janssens *et al.*, 2010). Although N additions have frequently been found to suppress heterotrophic respiration, the response of soil microbial communities is not yet well understood (Fog, 1988; Janssens *et al.*, 2010). Changes in microbial community composition following increased N deposition were often observed and ultimately affect key processes of the soil carbon (C) cycle which have ecosystem-level implications (Zak *et al.*, 2011). It is hypothesized that N deposition causes a shift from fungal- to bacterial-dominated microbial communities (Strickland & Rousk, 2010). Possible reasons are inhibition of fungal enzymes by N deposition (Fog, 1988), and outcompeting of less efficient fungi that require less N by highly efficient, but N limited, bacteria (Ågren *et al.*, 2001). This might be due to stoichiometric differences between fungi and bacteria, because fungi have higher C/N ratios than bacteria and therefore are expected to have lower N demands. Consequently a shift towards bacterial biomass is expected when N limitation is reduced due to atmospheric N deposition while C sources remain equally accessible (Strickland & Rousk, 2010).

Amino sugars are components of microbial cell-walls, which are stabilized in soil after cell death and are therefore used as biomarkers to assess fungal and bacterial residues in soil (Parsons, 1981; Amelung, 2001; Joergensen & Wichern, 2008). Amino sugars were used as a proxy for both, living and dead microbial biomass in soil. However, the living microbial biomass contributes only a small amount to the total amino sugars found in soil (Guggenberger *et al.*, 1999). Therefore, amino sugars not only reflect the microbial community at the time point of sampling, but can also be used to monitor medium-to-long-term changes in microbial community (Glaser *et al.*, 2004). Although more than 26 amino sugars have been identified, only three amino sugars are found in considerable amounts in soil: glucosamine, galactosamine and muramic acid. Glucosamine mainly originates from

chitin of fungal cell-walls, which is a polymer of N-acetyl-glucosamine units. However it is also present in bacterial cell-walls as part of peptidoglycan, where N-acetyl-glucosamine is alternately linked with N-acetyl-muramic acid. Muramic acid is exclusively found in bacterial peptidoglycan, making it a highly specific biomarker for bacteria. Higher contents of peptidoglycan were found in gram-positive compared to gram-negative bacteria (Guggenberger *et al.*, 1999; Amelung, 2001). Although galactosamine was found in fungi and bacteria (Engelking *et al.*, 2007) and its origin is less clearly defined, it was often used as an indicator for bacterial tissue. The ratios of glucosamine-to-galactosamine and of glucosamine-to-muramic acid are frequently used indicators for the relative contribution of fungi versus bacteria to microbial residues in soil (Amelung, 2001). Although many studies used the composition of amino sugars as a proxy for microbial residues in soil, little is known about the formation and stabilization of amino sugars in soil (Joergensen & Wichern, 2008). Recently developed compound-specific stable-isotope-analysis of individual amino sugars offers the possibility to elucidate these processes (Bodé *et al.*, 2009).

Stabilization of organic matter in soil is often defined as protection of organic matter against decomposition and is considered to be mainly driven by three mechanisms: (i) selective preservation of organic matter, (ii) spatial inaccessibility of organic matter and (iii) association of organic matter with soil minerals (Sollins *et al.*, 1996; Six *et al.*, 2002; von Lützow *et al.*, 2006). Selective preservation of organic matter is based on the concept that certain “recalcitrant” organic compounds persist in soil due to their molecular properties (Kleber, 2010). Plant-derived lignin was thought to be “recalcitrant” and therefore more effectively stabilized in soil than “labile” compounds like microbially-derived sugars (Baldock & Skjemstad, 2000). Although this was often observed in short-term litter decomposition experiments, there is growing evidence that the long-term stabilization of soil organic matter is not governed by the selective preservation of “recalcitrant” organic matter (von Lützow *et al.*, 2006; Marschner *et al.*, 2008; Schmidt *et al.*, 2011; Dungait *et al.*, 2012). Spatial inaccessibility of organic matter from decomposition is attributed to occlusion of organic matter within soil aggregates. Aggregate occluded organic matter is protected from decomposition by reduced access of microbes and reduced diffusion of their enzymes into soil aggregates. Also the diffusion of oxygen into aggregates is reduced and further limits aerobic decomposition (von Lützow *et al.*, 2006). Association of organic matter with soil minerals is thought to stabilize organic matter more effectively than aggregation. Stabilization of organic matter is accomplished by interactions of organic matter with clay mineral surfaces and

complexation with ions of calcium, iron and aluminum (von Lützow *et al.*, 2006; Kögel-Knabner *et al.*, 2008).

Soil density fractionation is frequently used to study the stabilization mechanisms of organic matter by separating qualitatively distinct organic matter fractions that should correspond to different stabilization mechanisms (Christensen, 1992; Christensen, 2001; von Lützow *et al.*, 2007). Light density fractions mainly consist of recent, only partially decomposed organic matter that is either physically unprotected or occluded with soil aggregates, while heavy density fractions consist of more decomposed organic matter that is associated with soil minerals (Golchin *et al.*, 1994; Crow *et al.*, 2007). Therefore, C concentrations, C/N ratios and mean residence times of organic C usually decrease from light to heavy density fractions (John *et al.*, 2005; Dorodnikov *et al.*, 2011). The application of promising new analytical techniques like compound-specific stable-isotope-analysis of biomarkers in soil density fractions will provide new insights into stabilization processes of organic matter in soil (Glaser, 2005; Amelung *et al.*, 2008; Simpson & Simpson, 2012; Gleixner, 2013).

This study made use of model forest ecosystems that had been isotopically labeled with ^{13}C-depleted CO_2 and treated with two levels of N deposition. We investigated the composition and dynamics of total organic C and microbial residues in bulk soil and density fractions using compound-specific stable-isotope-analysis of individual amino sugars. By linking state-of-the-art biomarker analysis with soil density fractionation, we wanted to gain insight into the formation and stabilization of soil organic matter in general and of microbial residues in particular. More specifically, we tested the hypothesis that microbial residues are mainly stabilized in fine mineral soil fractions. Furthermore, we tested the hypotheses that increased N deposition decreases the contribution of fungal-derived residues in soil and that mainly “new”, experiment-derived, microbial residues are affected in contrast to “old”, pre-experimental microbial residues.

Materials and methods

Experimental setup

We analyzed archived soil samples from a combined N deposition and elevated CO_2 experiment that was conducted over four growing seasons between 1994 and 1998. Model forest ecosystems were established in 12 open-top chambers (Egli *et al.*, 1998). An acidic soil with sandy loamy texture (Haplic Alisol) was transferred from a natural beech-spruce forest site into gravitational lysimeters. Soils were planted with beech (*Fagus sylvatica*) and spruce (*Picea abies*) trees as well as five typical understory species (*Carex sylvatica*, *Geum urbanum*, *Ranunculus ficaria*, *Viola sylvatica*, *Hedera helix*). Beech trees were two to three years old and spruce trees were four years old at planting. Ecosystems were treated with ambient CO_2 (370 µmol mol^{-1}; $\delta^{13}C$ = -8.3 ‰) and elevated CO_2 (570 µmol mol^{-1}; $\delta^{13}C$ = -29.8 ‰) in combination with two levels of N fertilizer (low N: 7 kg NH_4NO_3-N ha^{-1} yr^{-1}; high N: 70 kg NH_4NO_3-N ha^{-1} yr^{-1}). The following four treatments were applied: ambient CO_2 + low N; elevated CO_2 + low N; ambient CO_2 + high N; elevated CO_2 + high N. The treatments were arranged in a Latin square design with three replicates for each CO_2 x N treatment. After four years of treatment, soils were sampled from 0 to 10 cm depth prior to tree harvest. Soil samples were dried at 60 °C for 48 h immediately after sampling. Visible dead and living plant residues were removed from the soils by hand and soils were sieved through a 2 mm sieve. Samples were stored in a fully climatized archive (17 °C) with low air humidity (<40 %).

Soil fractionation

Density fractionation was carried out as described by Golchin *et al.* (1994). Bulk soil was separated into free light fraction (fLF), occluded light fraction (oLF) and heavy fraction (HF) by density fractionation and ultrasonic dispersion. HF material was further separated at 20 µm by particle-size fractionation (Fig. 1). The selection of density and dispersion energy should be based on preceding experiments, that show which parameters produce the maximum C content of light fractions (Cerli *et al.*, 2012; Griepentrog & Schmidt, 2013). We chose 1.6 g cm^{-3} as a density cut-off, because it was the most suitable density to separate maximum C content in light fractions (Cerli *et al.*, 2012). Dispersion energies should be determined for every soil, because they vary with aggregate stability. Therefore, we sequentially sonicated a soil sample to determine the dispersion energy which produces maximum C content of the occluded light fraction (Cerli *et al.*, 2012). Results showed that 250 J ml^{-1} was the most suitable dispersion energy for our soil.

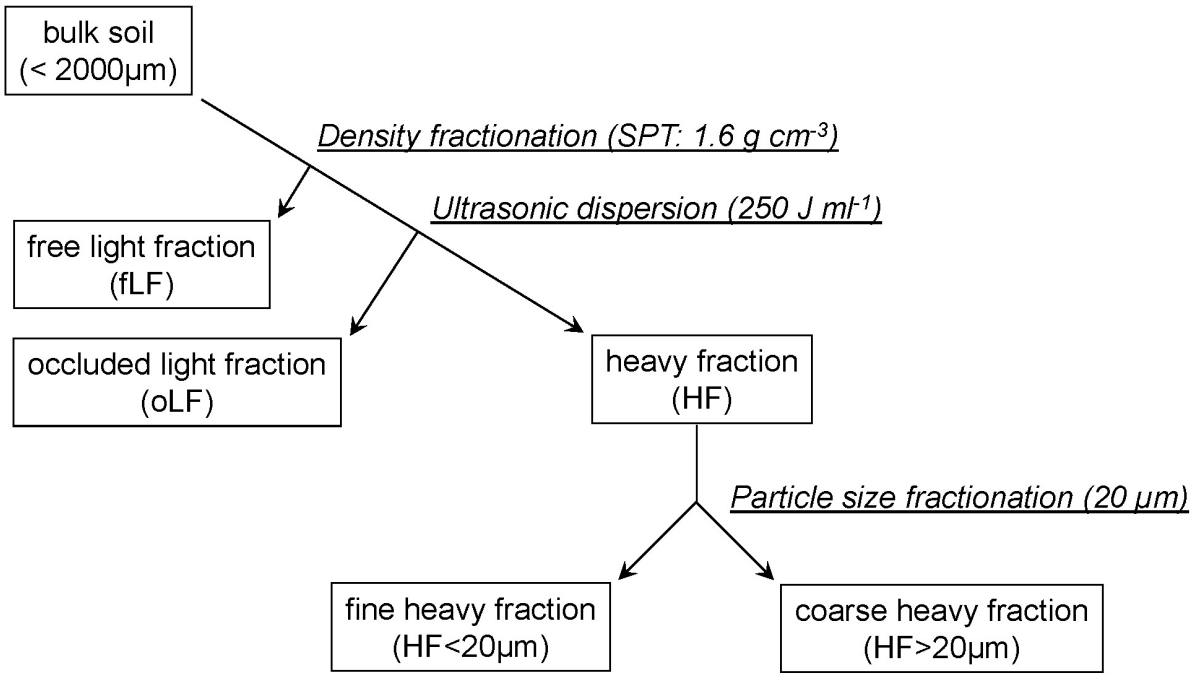

Figure 1 Soil fractionation scheme. Bulk soil was separated into free light fraction (fLF), occluded light fraction (oLF) and heavy fraction (HF) by density fractionation and ultrasonic dispersion. Heavy fraction was further particle size-fractionated at 20 µm into a coarse (HF>20µm) and a fine textured heavy fraction (HF<20µm).

Sodiumpolytungstate (*SPT 0, TC Tungsten Compounds, Grub am Forst, Germany*) solution was adjusted to a density of 1.6 g cm^{-3} by weighing out defined volumes of the solution. During the whole procedure, SPT solutions were collected and recycled with the method described by Six *et al.* (1999). Dried and sieved (<2 mm) bulk soil samples were suspended in SPT solution (soil:solution ratio 1:5) and subsequently centrifuged at 4500 g for 10 min. fLF material was collected by careful removal of the floating material and subsequent filtration using glass fibre filters (*GF 6, Whatman, Dassel, Germany*). fLF material was rinsed with deionized water until the electrical conductivity was <50 µS cm^{-1}, to remove residual SPT. The remaining soil material was resuspended in SPT solution (density 1.6 g cm^{-3}, soil:solution ratio 1:5) and ultrasonically dispersed with 250 J ml^{-1}. The ultrasonic equipment (*UW 3400, Bandelin, Berlin, Germany*) was calorimetrically calibrated according to Schmidt *et al.* (1999). oLF material was collected after centrifugation by careful removal of the floating material and filtration using glass fibre filters. oLF material were rinsed with deionized water until the electrical conductivity was <50 µS cm^{-1}, to remove residual SPT. The remaining soil material with a density >1.6 g cm^{-3} (HF) was washed three times with deionized water following centrifugation each time. HF material was further separated at 20 µm by wet sieving. The coarse heavy fraction larger 20 µm (HF>20µm) was collected from the sieve,

while the fine heavy fraction smaller 20 µm (HF<20µm) was collected after sedimentation. All fractions were freeze dried and milled prior to analysis. All soil samples were fractionated in triplicate and fractions were pooled for further analysis. On average, we recovered >99 % of the initial sample masses.

C / N (isotope) analysis

The C and N contents and the $\delta^{13}C$ values of bulk soil and soil density fractions were determined with an automated element analyzer - continuous flow isotope ratio mass spectrometer (*EA-1110, Carlo Erba, Fisons, Italy,* interfaced with a *ConFlo II* to a *Delta-S, Thermo Finnigan MAT, Bremen, Germany*). Results of the C isotope analysis are expressed in δ units (‰):

$$\delta^{13}C(‰) = 1000 \cdot \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \quad (1)$$

where R = ^{13}C / ^{12}C for both, sample and standard. The Vienna Pee Dee Belemnite (VPDB) standard was used as reference. Fraction of new soil C, derived from plant input during the experimental period was calculated from $\delta^{13}C$ values using a simple mixing model equation (Balesdent *et al.*, 1988):

$$F_{new} = \frac{\delta_{soil,depleted} - \delta_{soil,ambient}}{\delta_{plant,depleted} - \delta_{plant,ambient}} \quad (2)$$

where $\delta_{soil,depleted}$ and $\delta_{soil,ambient}$ are the $\delta^{13}C$ values of bulk soil or soil fractions for treatments with ^{13}C-depleted CO_2 and ambient CO_2, respectively. $\delta_{plant,depleted}$ and $\delta_{plant,ambient}$ are the $\delta^{13}C$ values of plant litter input for treatments with ^{13}C-depleted CO_2 and ambient CO_2, respectively. Concentrations of total organic C in the control treatments are within the range of that from before the experiment (Hagedorn *et al.*, 2001) and therefore the assumptions of Balesdent *et al.* (1988) should be valid for our calculation.

As the input of aboveground versus belowground plant litter into soil was not known, we took the mean of the $\delta^{13}C$ values of leaves, needles and fine roots for δ_{plant}. This assumption is reasonable, since the differences between $\delta^{13}C$ values of leaves, needles and fine roots were negligible compared to the large shift in $\delta^{13}C$ values due to four years of treatment with ^{13}C-

depleted CO_2. Plant biomass grown under ^{13}C-depleted CO_2 and ambient CO_2 differed on average by 10.5 ‰ in their $\delta^{13}C$ values (Hagedorn *et al.*, 2001). Treatments with elevated CO_2 did not significantly affect total organic C concentrations in bulk soil and soil fractions compared to treatments with ambient CO_2 (data not shown).

Amino sugar (isotope) analysis

Extraction of amino sugars from bulk soil and soil density fractions was adapted from the method described by Zhang & Amelung (1996). Therefore, amounts of sample material containing 0.3 mg of N were hydrolyzed by adding 6 M HCl (20 ml per gram of sample material) and heating at 105 °C for 8 h. Samples were filtered over glass fiber filters (*GF/C, Whatman, Dassel, Germany*) and the filtrate was evaporated to dryness at 40 to 45 °C under reduced pressure to remove HCl. Dried filtrate was redissolved in Milli-Q water (*Direct-Q 3 System, Millipore, Billerica, MA, USA*), transferred in a 2 ml tube (*Eppendorf, Hamburg, Germany*) and centrifuged. The supernatant was added onto a cation exchange resin (*AG 50W-X8, Bio-Rad Laboratories, Hercules, CA, USA*). After rinsing the resin with Milli-Q water to remove neutral and negatively charged compounds, the fraction containing amino sugars was eluted with 0.5 M HCl and again evaporated to dryness to remove HCl. Dried amino sugars were redissolved in Milli-Q water and transferred in a 2 ml tube. After desiccation using a centrifugal vacuum concentrator (*SpeedVac, Thermo Scientific, Langenselbold, Germany*), samples were stored at -18 °C until analysis.

Compound-specific stable-isotope-analysis of amino sugar extracts was done according to the method described by Bodé *et al.* (2009). Therefore, we used a high pressure liquid chromatography (HPLC) system existing of an autosampler (*Surveyor Autosampler Plus, Thermo Electron, Bremen, Germany*) and a HPLC pump (*Surveyor MS-Pump Plus, Thermo Electron*) with an analytical anion-exchange column (*PA20 CarboPac, 3 x 150 mm, 6.5 µm*) that was coupled through a wet oxidation interface (*LC Isolink, Thermo Electron*) to an isotope ratio mass spectrometer (IRMS; *DELTA^{PLUS} XP, Thermo Elec*tron).

Bacterial- and fungal-derived amino sugar C was calculated according to Van Groenigen *et al.* (2007). Based on the assumption that all galactosamine and muramic acid are produced by bacteria and that glucosamine and muramic acid occur in equal amounts in bacteria

$$\text{Bacterial AS-C} = \text{galactosamine C} + 2 \cdot \text{muramic acid C} \quad (3)$$

The remainder of the amino sugar C pool is derived from fungi and therefore

$$\text{Fungal AS-C} = \text{glucosamine C} - \text{muramic acid C} \quad (4)$$

Treatments with elevated CO_2 did not significantly affect amino sugar concentrations in bulk soil and soil fractions compared to treatments with ambient CO_2 (data not shown).

Statistical analysis

For replicate measurements the mean of three field replicates is given along with the standard error (Webster, 2001). Effects of CO_2 treatment, N deposition and their interactions were tested for significant differences between and within fractions and bulk soil by analysis of variance (Webster, 2007).

Results and discussion

Concentrations of organic carbon and nitrogen

Highest C concentrations were found in both light fractions, while occluded light fractions showed slightly higher C concentrations than free light fractions (Table 1). Both light fractions together stored 29% of the soil organic C, whereof around three quarters were stored in free light fractions and one quarter in occluded light fractions (Fig. 2a). Heavy fractions had substantially lower C concentrations than light fractions. Here, fine heavy fractions showed higher C concentrations than coarse heavy fractions (Table 1). Most of the soil organic C was recovered in fine heavy fractions (62 %), while coarse heavy fractions contributed only 9 % to soil organic C (Fig. 2a). C/N ratios were highest in both light fractions, decreased in coarse heavy fraction and were lowest in the fine heavy fraction (Table 1).

High C concentrations and C/N ratios in light density fractions are commonly observed, showing that they consist of fresh organic matter, which is in agreement with the concept of the density fractionation approach (Cerli *et al.*, 2012). Organic matter in free light fractions mainly originates from plant residues, while organic matter in occluded light fractions consists of more fragmented plant residues and materials with less recognizable structure, indicating that organic matter in occluded light fractions is more degraded than in free light fractions (Golchin *et al.*, 1994; Crow *et al.*, 2007; Wagai *et al.*, 2009; Dorodnikov *et al.*, 2011). Higher C concentrations in occluded compared to free light fractions can be attributed to increased protection of organic matter from degradation due to aggregation and were also observed by other studies (Golchin *et al.*, 1994; John *et al.*, 2005; Dorodnikov *et al.*, 2011).

Lower C concentrations and C/N ratios in heavy fractions compared to light fractions are attributed to an increasing degree of organic matter degradation and incorporation of organic matter into microbes (John *et al.*, 2005). Higher C concentrations in fine heavy fractions compared to coarse heavy fractions are attributed to enhanced protection of organic matter by mineral surfaces in the fine heavy fractions and interactions with clay minerals and Fe and Al oxides are thought to be most relevant for organic matter stabilization (Kögel-Knabner *et al.*, 2008). Several studies found that a major part of soil organic C was associated with heavy fractions (John *et al.*, 2005; Dorodnikov *et al.*, 2011).

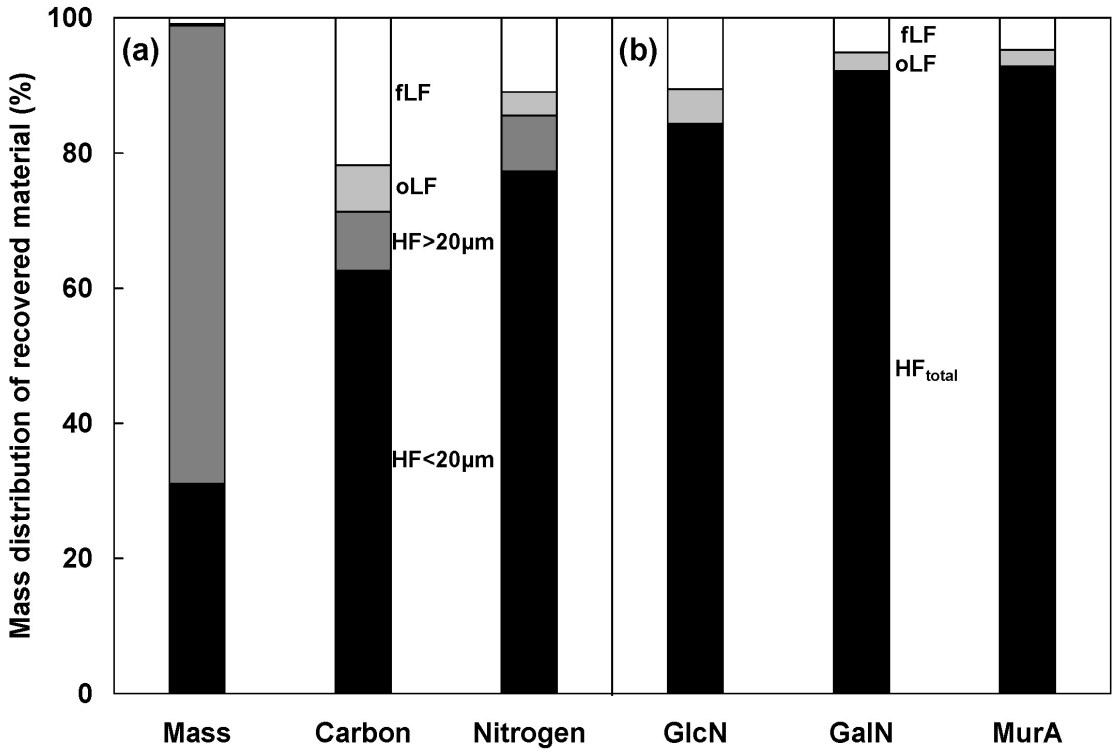

Figure 2 Mass distribution of recovered (a) soil mass, total organic carbon, total nitrogen and (b) amino sugars (glucosamine, GlcN; galactosamine, GalN; muramic acid, MurA) between soil fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, HF_{total}; coarse heavy fraction, HF>20µm; fine heavy fraction, HF<20µm). Recoveries after fractionation procedure were (a) 99.2 ± 0.1 % of mass, 89.3 ± 1.8 % of carbon, 87.9 ± 1.6 % of nitrogen and (b) 94.5 ± 2.8 % of GlcN, 90.4 ± 4.6 % of GalN, 67.9 ± 8.9 % MurA. For amino sugars (b), coarse heavy fraction was not analyzed and therefore only total heavy fraction is shown. Average data for the four experimental treatments is shown.

Increased N deposition decreased the C/N ratios of both light fractions (Table 1). In free light fractions the decrease in C/N was caused by a significant increase in N concentrations (Table 1), very likely reflecting higher N concentrations and decreased C/N ratios after N additions in foliage (Hagedorn *et al.*, 2000) and fine roots (Hagedorn *et al.*, 2001) of this experiment. In contrast to free light fractions, the decrease in C/N ratio in occluded light fractions was the consequence of a significant decrease in C concentrations (Table 1). However, also more material was recovered and thus the total C pool of occluded light fractions did not change for both N treatments.

Table 1 Mass, organic carbon (C), total nitrogen (N), and C/N ratios in bulk soil and soil fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, HF; coarse heavy fraction, HF>20µm; fine heavy fraction, HF<20µm) for treatments with low and high N deposition. Total C was separated into new (experiment-derived) C and old (pre-experiment) C using isotope mixing model. Asterisks denote significant N treatment effects: n.s. = not significant (p>0.05), * p<0.05, ** p<0.01, *** p<0.001.

	Mass	Organic carbon ($gC_{fr}\ kg_{fr}^{-1}$)			Nitrogen	C / N
	($g_{fr}\ kg_{soil}^{-1}$)	total C	new C	old C	($gN_{fr}\ kg_{fr}^{-1}$)	(-)
Bulk soil	n.s.	n.s.	n.s.	n.s.	n.s.	*
Low nitrogen	1000.0 ± 0.0	14.9 ± 0.5	3.2 ± 0.4	11.6 ± 0.4	1.0 ± 0.0	15.6 ± 0.3
High nitrogen	1000.0 ± 0.0	14.7 ± 0.4	3.1 ± 0.2	11.6 ± 0.2	1.0 ± 0.0	14.7 ± 0.2
Density fractionation						
Free light fraction (fLF)	n.s.	n.s.	n.s.	n.s.	***	***
Low nitrogen	8.7 ± 0.4	323.6 ± 4.7	192.0 ± 19.4	131.6 ± 19.4	9.8 ± 0.1	33.2 ± 0.8
High nitrogen	9.0 ± 0.4	326.6 ± 6.9	195.2 ± 14.0	131.4 ± 14.0	11.6 ± 0.2	28.2 ± 0.6
Occluded light fraction (oLF)	n.s.	*	n.s.	n.s.	n.s.	***
Low nitrogen	2.3 ± 0.2	393.1 ± 10.3	83.4 ± 13.6	309.7 ± 13.6	12.1 ± 0.2	32.4 ± 0.7
High nitrogen	2.6 ± 0.1	353.1 ± 8.3	73.4 ± 10.7	279.8 ± 10.7	12.4 ± 0.2	28.4 ± 0.4
Total heavy fraction (HF)	*	n.s.	n.s.	n.s.	**	*
Low nitrogen	979.2 ± 0.8	9.5 ± 0.1	1.0 ± 0.1	8.5 ± 0.1	0.7 ± 0.0	13.0 ± 0.1
High nitrogen	982.8 ± 0.9	9.7 ± 0.2	1.3 ± 0.2	8.4 ± 0.2	0.8 ± 0.0	12.6 ± 0.1
Particle-size fractionation						
Coarse heavy fraction (HF>20µm)	n.s.	n.s.	*	n.s.	n.s.	n.s.
Low nitrogen	669.8 ± 2.9	1.4 ± 0.0	0.2 ± 0.0	1.2 ± 0.0	0.1 ± 0.0	14.0 ± 0.4
High nitrogen	661.7 ± 2.6	1.6 ± 0.1	0.4 ± 0.0	1.1 ± 0.0	0.1 ± 0.0	15.5 ± 0.7
Fine heavy fraction (HF<20µm)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Low nitrogen	301.8 ± 3.3	22.9 ± 0.5	1.9 ± 0.2	20.9 ± 0.2	2.0 ± 0.1	11.2 ± 0.2
High nitrogen	308.2 ± 3.0	23.3 ± 0.3	2.5 ± 0.2	20.8 ± 0.2	2.1 ± 0.0	11.3 ± 0.1

Isotopic composition of total organic carbon

Density fractionation separated individual soil fractions with significantly different C isotopic composition. Under both, ambient and ^{13}C-depleted CO_2 treatments, ^{13}C abundance increased in the order: free light fraction < occluded light fraction < coarse heavy fraction < fine heavy fraction (Fig. 3), as observed by other studies (John *et al.*, 2005; Dorodnikov *et al.*, 2011). Organic C in bulk soil is generally enriched in ^{13}C compared to that in light fractions, indicating degradation of plant residues (Ehleringer *et al.*, 2000). Enrichment of organic matter in ^{13}C is generally attributed to isotope fractionation during microbial degradation or preferential substrate utilization by microorganisms (Blagodatskaya *et al.*, 2011), but also to gradual shifts in the relative contribution of microbial versus plant components (Ehleringer *et al.*, 2000). Using ^{13}C CP/MAS NMR, Golchin *et al.* (1994) could also show that occluded light fractions and heavy fractions contained organic matter that was further degraded than in free light fractions.

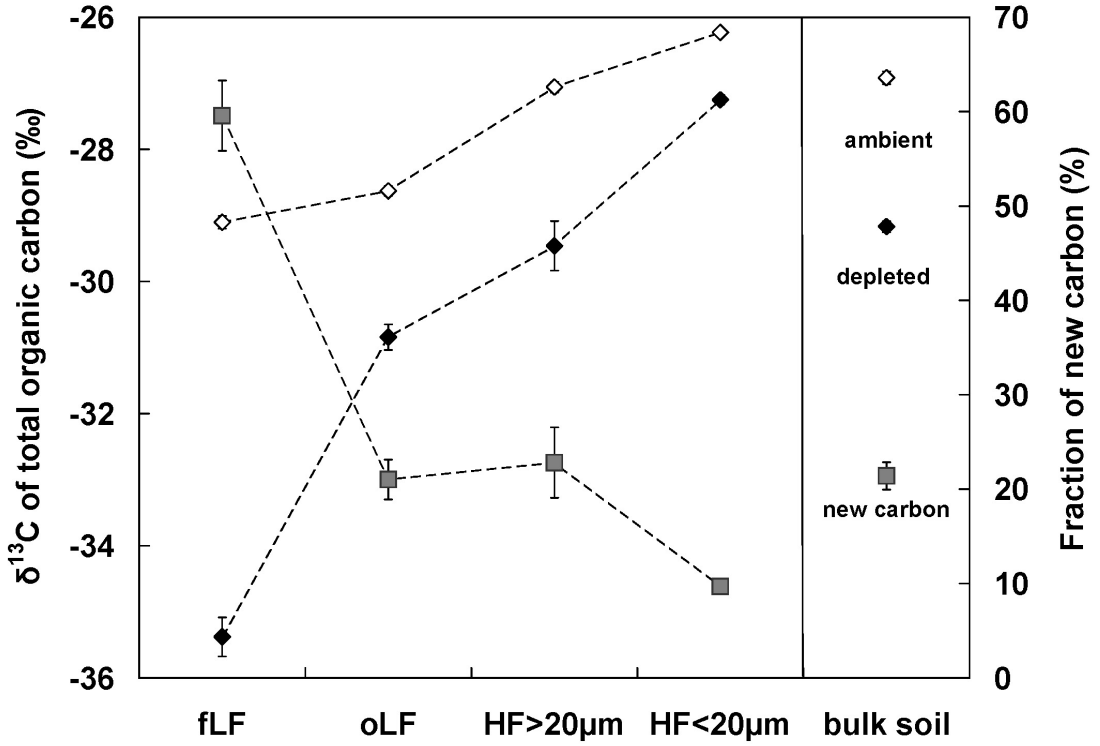

Figure 3 Carbon isotope ratios ($\delta^{13}C$) of total organic carbon in bulk soil and soil fractions (free light fraction, fLF; occluded light fraction, oLF; coarse heavy fraction, HF>20µm; fine heavy fraction, HF<20µm) for treatments with ambient and ^{13}C-depleted CO_2 as well as the fraction of new carbon synthesized during four years of experiment. Average data for the two nitrogen deposition treatments is shown.

Four years of treatment with ^{13}C-depleted CO_2 significantly decreased $\delta^{13}C$ values compared to treatment with ambient CO_2 for bulk soil (-2.3 ± 0.1 ‰) and individual density fractions (Fig. 3). The difference in $\delta^{13}C$ values between treatments with ambient and ^{13}C-depleted CO_2 is a measure for the incorporation of isotopic label during the experimental period and hence for the fraction of "new" plant-derived C. Since free light fractions mainly consist of recent plant residues, they had the highest percentage of newly formed organic matter (60 %). Occluded light fractions contained considerably less organic matter from the experimental period (21 %), showing that the transfer of organic matter from free light to occluded light fractions and the replacement of the organic matter pool in occluded light fractions is rather slow. This might be due to the stabilization of organic matter by aggregation in occluded light fractions (Sollins *et al.*, 1996; Six *et al.*, 2002). The percentage of new C was not significantly different between occluded light and coarse heavy fractions. This could be due to a rather slow replacement of the organic matter pool in coarse heavy fractions or a fast transfer of

organic matter from occluded light to coarse heavy fractions. Due to continuous renewal of soil aggregates (Six *et al.*, 2000) a close interaction between occluded materials and coarse mineral fraction can be assumed and therefore an equal stabilization of organic matter in both fractions. Fine heavy fractions showed the lowest percentage of new organic matter (10 %). Here, the slow replacement of the organic matter pool is attributed to the large C pool in this fraction (Fig. 2a) and also to the strong interaction between organic matter and soil minerals, which was shown to be a major control of organic matter stabilization in soils (Kögel-Knabner *et al.*, 2008).

Increased N deposition significantly increased the amount of new, experiment-derived C in coarse heavy fractions (Table 1). However, due to the small amount of C stored in coarse heavy fractions (Fig. 2a), this increase might not be relevant for total soil C sequestration. The result is consistent with a study from the same experiment, that also found significantly greater amounts of new C in coarse sand fractions after particle-size fractionation (Hagedorn *et al.*, 2003). Nevertheless, increased N deposition did not affect the amounts of old versus new C in the other soil fractions significantly (Table 1). This shows that increased N deposition did not substantially alter how new and old C was distributed between the different soil fractions in our study. In contrast to our study, Hagedorn *et al.* (2003) found after particle-size fractionation, that increased N deposition reduced the amount of old C in silt and clay fractions that was mineralized during the experimental period. In line, Neff *et al.* (2002) also observed that N additions significantly increased C in mineral-associated soil fractions. Mechanisms that could explain the retarded decomposition of old C under increased N deposition are changes in the microbial decomposer community (Janssens *et al.*, 2010) or a preferential substrate use of decomposer organisms (Liljeroth *et al.*, 1990).

Concentrations of individual amino sugars

Total amino sugar C contributed 1.4 % to total soil organic C (Fig. 4a). In bulk soil and density fractions, the concentrations of individual amino sugars decreased in the order: glucosamine > galactosamine > muramic acid (Fig. 4a). In bulk soil, glucosamine, galactosamine and muramic acid contributed 63 %, 26 % and 11 % to the total amino sugar C, respectively. Similar amounts of total amino sugars and a comparable distribution of individual amino sugars are typically observed (Van Groenigen *et al.*, 2007; Van Groenigen *et al.*, 2010; Bai *et al.*, 2013). Individual amino sugar concentrations differed significantly among density fractions and increased in the order: free light fraction < occluded light fraction < coarse heavy fraction < fine heavy fraction (Fig. 4a). Also on a mass balance, most

amino sugars were recovered in heavy fractions. 84 %, 92 %, and 97 % of glucosamine, galactosamine and muramic acid were found in heavy fractions, respectively (Fig. 2b). Our results are in agreement with other studies showing that amino sugar concentrations increased from coarse to fine fractions and that most of the total amino sugars are found in silt- and clay-sized fractions (Zhang *et al.*, 1999; Solomon *et al.*, 2001; Turrión *et al.*, 2002). Also analysis of non-cellulosic neutral sugars indicated that microbially-derived sugars are accumulating in heavy density fractions (Rumpel *et al.*, 2010) and fine particle-size fractions (Kiem & Kögel-Knabner, 2003; Jolivet *et al.*, 2006; Spielvogel *et al.*, 2008). The high amino sugar concentrations found in the heavy fractions further corroborate that microbial residues are typically associated to fine-textured soil minerals. Therefore, association with soil minerals seems to be the most significant stabilization mechanism for microbial residues in soil (Guggenberger *et al.*, 1999).

Both amino sugar ratios, glucosamine-to-galactosamine and glucosamine-to-muramic acid, decreased in the order: free light fraction > occluded light fraction > total heavy fraction > fine heavy fraction (Fig. 4b). Changes in amino sugars ratios of glucosamine-to-galactosamine and of glucosamine-to-muramic acid are used to qualitatively determine relative increases or decreases of fungal versus bacterial residues. However, due to the unspecific origin of glucosamine, changes of both ratios should be consistent (Amelung, 2001). In our study, both fungi-to-bacteria-ratios consistently decreased by a factor of 3 from free light to fine heavy fractions, indicating a relative shift from fungal to bacterial residues in this direction. Another way to express the contribution of fungal- versus bacterial-derived residues is based on the stoichiometric differences between fungal-derived chitin and bacterial-derived peptidoglycan. The calculation is based on the assumptions that galactosamine is solely originating from bacteria, while bacterial glucosamine is solely originating from peptidoglycan and that glucosamine and muramic acid occur in a constant ratio in peptidoglycan (Van Groenigen *et al.*, 2007). Here, fungal- and bacterial-derived amino sugar C both increase from free light to fine heavy fractions, while the fungal-to-bacterial ratio decreases in the same direction (Fig. 5), which is in line with the observation from glucosamine-to-galactosamine and glucosamine-to-muramic acid ratios (Fig. 4b) and further corroborates the relative enrichment of bacterial residues in fine heavy fractions. In line, other studies on amino sugars observed that fungal-to-bacterial ratios decreased from coarse sand to clay fractions (Zhang *et al.*, 1999; Solomon *et al.*, 2001; Turrión *et al.*, 2002). Potential reasons for preferential association of bacterial residues with soil minerals could be that in contrast to bacteria, most fungi are obligatory aerobes and are restricted to air-filled

spaces in soil. Fungal hyphae where found to dominate in the outer regions of macroaggregates, while bacteria dominate in the center of microaggregates (Chenu & Stotzky, 2002).

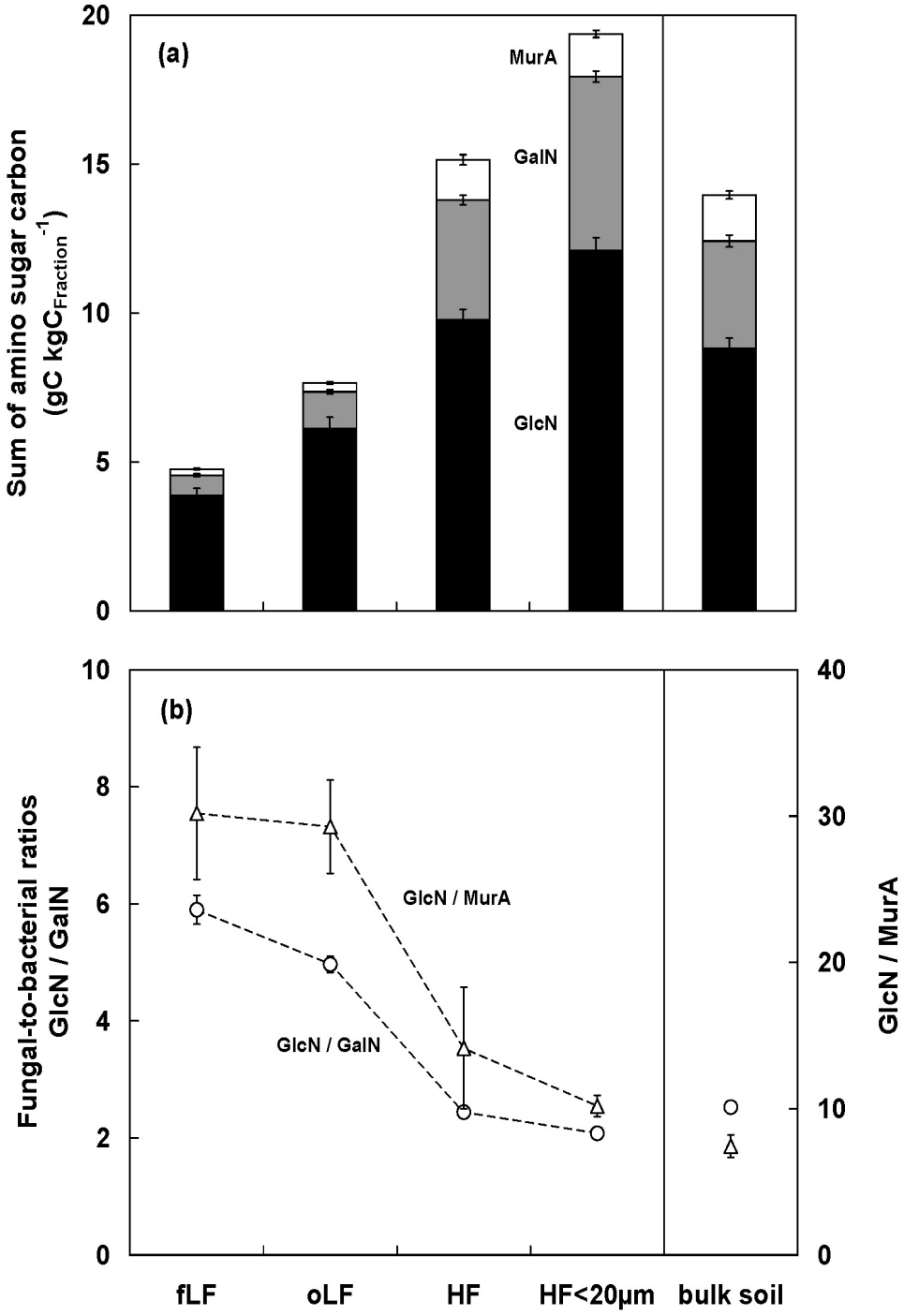

Figure 4 Amino sugar carbon normalized to total organic carbon (a: glucosamine, GlcN; galactosamine, GalN; muramic acid, MurA) and fungal-to-bacterial ratios (b: GlcN/GalN and GlcN/MurA) in bulk soil and soil fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, HF; fine heavy fraction, HF<20µm). Average data for the four experimental treatments is shown.

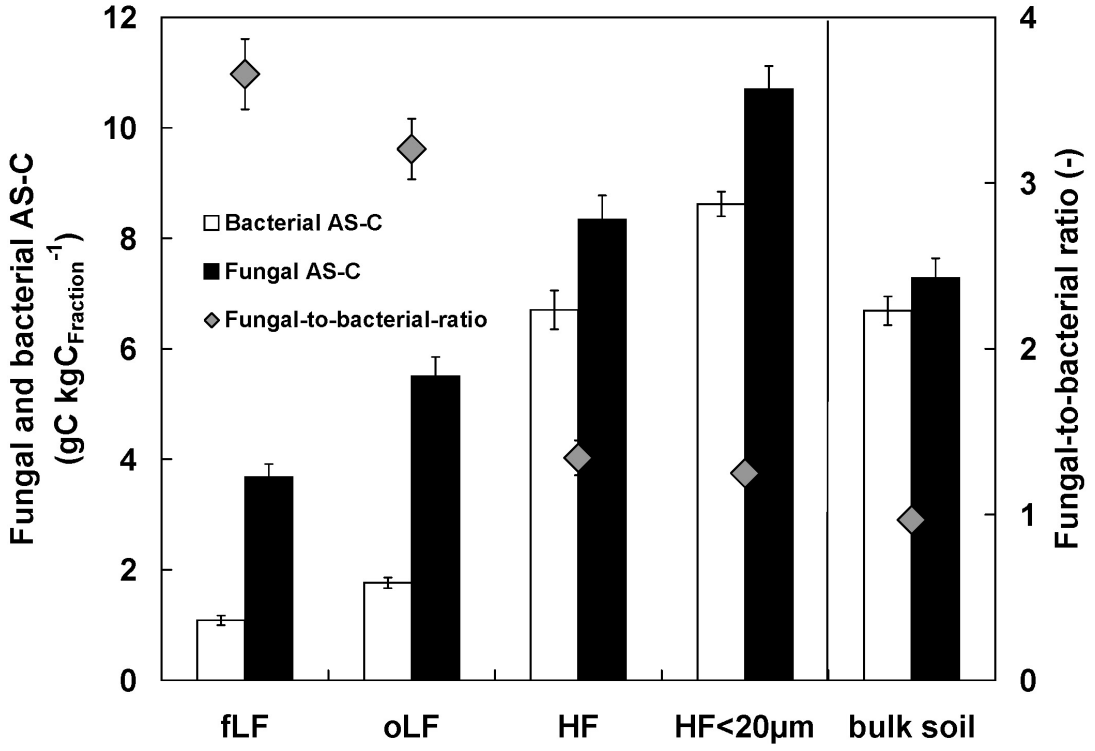

Figure 5 Fungal- and bacterial-derived amino sugar carbon (AS-C) as well as their ratio calculated after Van Groenigen *et al.* (2007) and normalized to total organic carbon in bulk soil and soil fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, HF; fine heavy fraction, HF<20µm). Average data for the four experimental treatments is shown.

Increased N deposition did significantly increase the total content of glucosamine in fine heavy fractions (Table 2) and consequently the total fungal-derived amino sugars in fine heavy fractions (Table 3). These results are in line with a previous study from the same experiment, showing that N deposition significantly increased fungal biomass (determined by ergosterol content) associated with fine roots and in root-free soil (Wiemken *et al.*, 2001a), while it did neither affected total microbial biomass (determined by fumigation extraction) nor phospholipid fatty acids (Wiemken *et al.*, 2001b). However, ergosterol and phospholipid fatty acids rapidly decompose after cell death and mainly represent the living microbial biomass at the time of sampling. In contrast, amino sugars are substantially stabilized in soil after death of microbial biomass (Glaser *et al.*, 2004) and therefore their abundance might be a more useful indicator for long-term microbial responses (Van Groenigen *et al.*, 2007). Our results in a forest soil contrast with the study by Van Groenigen *et al.* (2007) in a grassland ecosystem, where N addition had significant effects on amino sugars with high rates of N fertilizer decreasing fungal residues. However, their study lasted ten years and used a ten

times higher N input added in single high doses as compared to our study applying lower amounts of N (70 kg N ha^{-1} yr^{-1}) continuously. Nevertheless, a study in a forest ecosystem that used lower amounts of N (15 kg N ha^{-1} yr^{-1}) over a longer period (14 years) also showed that N additions resulted in a significant reduction of fungal biomass (Frey *et al.*, 2004). In contrast, Dörr *et al.* (2010) studied the impact of reduced N deposition in a N-saturated forest ecosystem and did not find changes in amino sugar composition after 14.5 years. Initial effects could be shown at the plant level, but Dörr *et al.* (2010) argued that the experimental period was too short to detect effects on soil organic matter, which generally has higher mean turnover times (Amelung *et al.*, 2008).

Our results could have been biased by drying the soils at 60 °C and storing them for 15 years before analysis. To our knowledge, there had not been a thorough assessment of long-term soil storage on amino sugars. However, the study of Zelles *et al.* (1991) showed that storing soils for 1, 2 and 20 months at different temperatures (+21, +4, -18 and -140 °C) did not have significant effects on amino sugar compositions (see statistical evaluation by Amelung, 2001). We also do not expect that the relatively high drying temperature of 60 °C affected our results, since amino sugar polymers are relatively stable (Amelung, 2001) and the differences among soil fractions were large and consistent with other studies conducted with fresh soil samples (e.g. Guggenberger *et al.*, 1999). However, the drying step could potentially have a beneficial effect with respect to sample storage, as it will degenerate most proteins and be fatal for large parts of the living soil microorganisms. Moreover, soil samples used in our study have been stored in a fully climatized archive at air humidity below 40%, which further prevents microbial activity. Finally, our measurements of C contents (Table 1) and $\delta^{13}C$ values (Figure 3) are well in line with that of Hagedorn *et al.* (2004) who measured the same samples about ten years ago, which indicates that sample storage had no major effects on organic matter composition in our soil samples.

Table 2 Amino sugar carbon (C) in bulk soil and soil fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, HF; fine heavy fraction, HF<20µm) normalized to total organic C content, for treatments with low and high nitrogen (N) deposition. Total C was separated into new (experiment-derived) C and old (pre-experiment) C using isotope mixing model. Asterisks denote significant N treatment effects: n.s. = not significant (p>0.05), * p<0.05, ** p<0.01.

	Glucosamine (gC kgC_{fr}^{-1})			Galactosamine (gC kgC_{fr}^{-1})			Muramic acid (gC kgC_{fr}^{-1})		
	total C	new C	old C	total C	new C	old C	total C	new C	old C
Bulk soil	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
Low nitrogen	8.4 ± 0.3	1.5 ± 0.3	6.9 ± 0.3	3.5 ± 0.3	0.2 ± 0.2	3.4 ± 0.2	1.5 ± 0.2	0.2 ± 0.0	1.3 ± 0.0
High nitrogen	9.2 ± 0.6	2.8 ± 0.3	6.4 ± 0.3	3.7 ± 0.3	0.4 ± 0.1	3.3 ± 0.1	1.6 ± 0.2	0.1 ± 0.0	1.5 ± 0.0
Density fractionation									
Free light fraction (fLF)	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
Low nitrogen	3.7 ± 0.4	2.2 ± 0.3	1.5 ± 0.3	0.6 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
High nitrogen	4.1 ± 0.3	2.3 ± 0.2	1.8 ± 0.2	0.7 ± 0.1	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Occluded light fraction (oLF)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Low nitrogen	5.9 ± 0.4	1.0 ± 0.3	4.9 ± 0.3	1.2 ± 0.1	0.2 ± 0.1	1.0 ± 0.1	0.3 ± 0.0	0.0 ± 0.1	0.2 ± 0.1
High nitrogen	5.7 ± 0.6	1.0 ± 0.6	4.8 ± 0.6	1.2 ± 0.1	0.3 ± 0.2	0.9 ± 0.2	0.3 ± 0.1	-0.1 ± 0.0	0.4 ± 0.0
Total heavy fraction (HF)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Low nitrogen	9.6 ± 0.5	1.3 ± 0.0	8.3 ± 0.0	4.1 ± 0.2	0.2 ± 0.2	3.9 ± 0.2	1.1 ± 0.2	0.0 ± 0.1	1.1 ± 0.1
High nitrogen	10.0 ± 0.5	1.4 ± 0.6	8.5 ± 0.6	3.9 ± 0.2	0.1 ± 0.1	3.8 ± 0.1	1.6 ± 0.3	0.4 ± 0.1	1.2 ± 0.1
Particle-size fractionation									
Fine heavy fraction (HF<20µm)	**	n.s.	*	n.s.	n.s.	*	n.s.	n.s.	n.s.
Low nitrogen	11.2 ± 0.5	1.8 ± 0.3	9.4 ± 0.3	5.5 ± 0.3	0.4 ± 0.2	5.1 ± 0.2	1.5 ± 0.2	0.1 ± 0.2	1.3 ± 0.2
High nitrogen	13.1 ± 0.4	1.7 ± 0.5	11.4 ± 0.5	6.0 ± 0.2	0.1 ± 0.1	5.8 ± 0.1	1.4 ± 0.2	0.1 ± 0.1	1.4 ± 0.1

Table 3 Fungal- and bacterial-derived amino sugar (AS) carbon (C) in bulk soil and soil fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, HF; fine heavy fraction, HF<20µm) normalized to total organic C content, for treatments with low and high nitrogen (N) deposition. Fungal and bacterial AS-C was calculated after Van Groenigen *et al.* (2007). Total C was separated into new (experiment-derived) C and old (pre-experiment) C using isotope mixing model. Asterisks denote significant N treatment effects: n.s. = not significant (p>0.05), * p<0.05, ** p<0.01.

	Fungal AS-C (gC kgC_{fr}^{-1})			Bacterial AS-C (gC kgC_{fr}^{-1})		
	total C	new C	old C	total C	new C	old C
Bulk soil	n.s.	*	n.s.	n.s.	n.s.	n.s.
Low nitrogen	7.0 ± 0.4	1.3 ± 0.3	5.6 ± 0.3	6.5 ± 0.4	0.5 ± 0.1	6.0 ± 0.1
High nitrogen	7.6 ± 0.6	2.7 ± 0.3	5.0 ± 0.3	6.9 ± 0.4	0.6 ± 0.2	6.3 ± 0.2
Density fractionation						
Free light fraction (fLF)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Low nitrogen	3.5 ± 0.3	2.1 ± 0.2	1.4 ± 0.2	1.1 ± 0.1	0.4 ± 0.1	0.7 ± 0.1
High nitrogen	3.9 ± 0.1	2.2 ± 0.2	1.6 ± 0.2	1.1 ± 0.0	0.3 ± 0.1	0.8 ± 0.1
Occluded light fraction (oLF)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Low nitrogen	5.6 ± 0.3	0.9 ± 0.3	4.7 ± 0.3	1.7 ± 0.1	0.3 ± 0.2	1.4 ± 0.2
High nitrogen	5.4 ± 0.5	1.0 ± 0.6	4.4 ± 0.6	1.8 ± 0.2	0.2 ± 0.3	1.7 ± 0.3
Total heavy fraction (HF)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Low nitrogen	8.5 ± 0.6	1.2 ± 0.1	7.3 ± 0.1	6.3 ± 0.4	0.3 ± 0.2	6.0 ± 0.2
High nitrogen	8.4 ± 0.7	1.0 ± 0.8	7.3 ± 0.8	7.1 ± 0.3	0.9 ± 0.2	6.2 ± 0.2
Particle-size fractionation						
Fine heavy fraction (HF<20µm)	**	n.s.	*	n.s.	n.s.	n.s.
Low nitrogen	9.8 ± 0.3	1.7 ± 0.4	8.1 ± 0.4	8.4 ± 0.2	0.7 ± 0.5	7.6 ± 0.5
High nitrogen	11.7 ± 0.5	1.6 ± 0.5	10.1 ± 0.5	8.8 ± 0.3	0.2 ± 0.1	8.6 ± 0.1

Carbon isotopic composition of individual amino sugars

In bulk soil and density fractions, amino sugars were enriched in ^{13}C compared to total organic C at natural abundance. Enrichment of ^{13}C increased in the order: total organic C < glucosamine < galactosamine < muramic acid (Fig. 6a,b). Differences between $\delta^{13}C$ values of total organic C and microbial residues result from isotopic fractionation during the biochemical synthesis of individual compounds (e.g. amino sugars) and from different $\delta^{13}C$ values of the substrates used for biosynthesis (Hobbie *et al.*, 1999). Gleixner *et al.* (1993) found that fungi were 4 ‰ enriched in ^{13}C compared to their substrates in wood. Chitin in their cell walls was enriched by 2 ‰ relative to wood cellulose. They further argued that the hexose units of chitin (amino sugars) must be even more enriched in ^{13}C, because the polymer has substitution by acetyl groups, which should be ^{13}C-depleted (Melzer & Schmidt, 1987). These findings are in line with our observation that microbial residues are generally enriched in ^{13}C compared to total organic C for both, ambient and ^{13}C-depleted CO_2 treatments. In contrast to our results and the results presented above, Glaser *et al.* (2006) determined ^{13}C isotopic signatures of individual amino sugars in a grassland ecosystem and found that galactosamine was depleted in ^{13}C compared to total organic C. However, the applicability of their method (GC-IRMS) to determine $\delta^{13}C$ values of amino sugars in soil at natural abundances was recently questioned, because of high analytical errors during derivatization (Decock *et al.*, 2009).

Among soil fractions, C isotope ratios of individual amino sugars showed the same pattern as total organic C. Amino sugars in heavy fractions were enriched in ^{13}C compared to amino sugars in light fractions (Fig. 6a,b). ^{13}C enrichment of amino sugars in heavy fractions is possibly attributed to isotope fractionation during decomposition of amino sugars, a mechanism which is frequently discussed for organic matter in general (Galimov, 2006). This would suggest that amino sugars in heavy fractions are decomposed to a higher proportion than in light fractions. Another possibility for the ^{13}C enrichment of amino sugars in heavy fractions is that the C source for amino sugar formation is taken from the ^{13}C-enriched C already present in the fraction.

Adding ^{13}C-depleted CO_2 for four years significantly decreased $\delta^{13}C$ values of amino sugars compared to treatments with ambient CO_2 (Fig. 6a,b). The fraction of newly formed amino sugars followed the pattern of total organic C, with the highest fraction of new C in free light fractions and considerably lower fractions of new C in occluded light and heavy fractions (Fig. 7). This suggests that the replacement of amino sugar pools in these fractions is slower than in free light fractions, which might be due to stabilization of amino sugars by

aggregation in occluded light fractions and association with soil minerals in fine heavy fractions. However, also the total pool size of amino sugars is larger in heavy than in light fractions and hence it takes much longer until this pool is replaced. Nevertheless, we do not know, where new amino sugars are actually formed, since they could be formed "in situ" within soil fractions, but also transferred between fractions. We also do not know on which timescales the "in-situ" formation of amino sugars in soil fractions and the transfer of amino sugars between soil fractions occur.

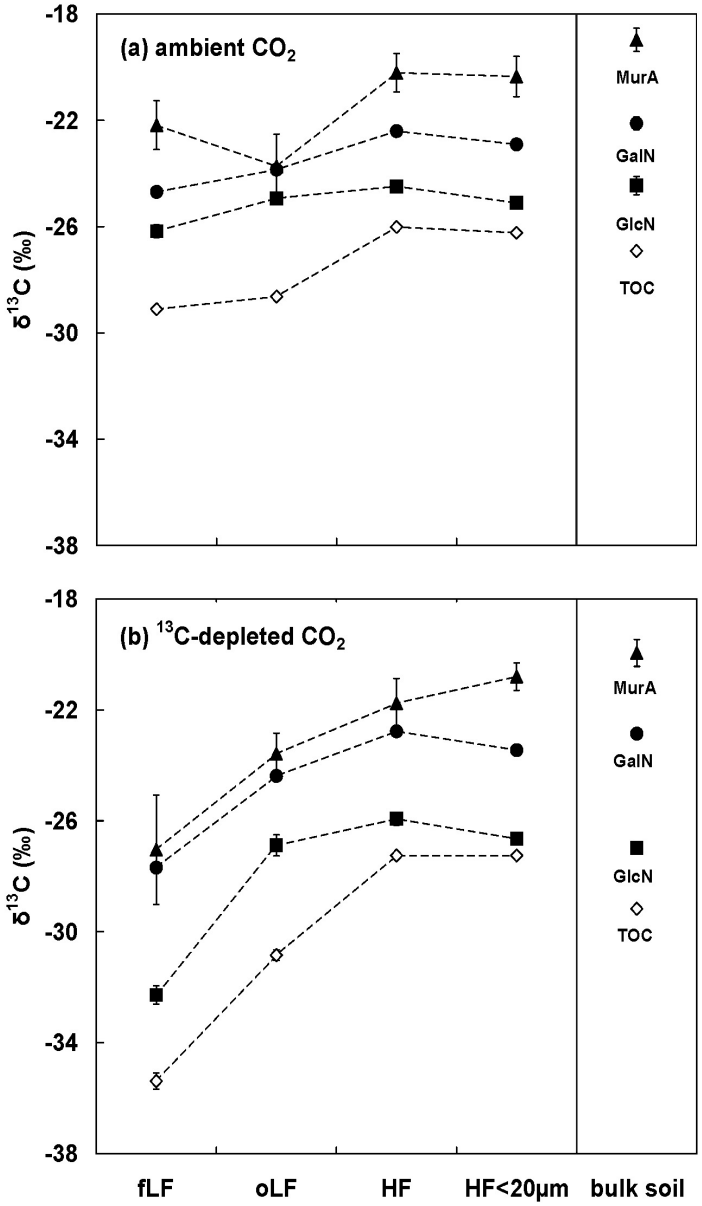

Figure 6 Carbon isotope ratios ($\delta^{13}C$) of total organic carbon (TOC) and individual amino sugars (glucosamine, GlcN; galactosamine, GalN; muramic acid, MurA) in bulk soil and soil fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, HF; fine heavy fraction, HF<20µm) for treatments with (a) ambient CO_2 and (b) ^{13}C-depleted CO_2 after four years of experiment. Average data for the two nitrogen deposition treatments is shown.

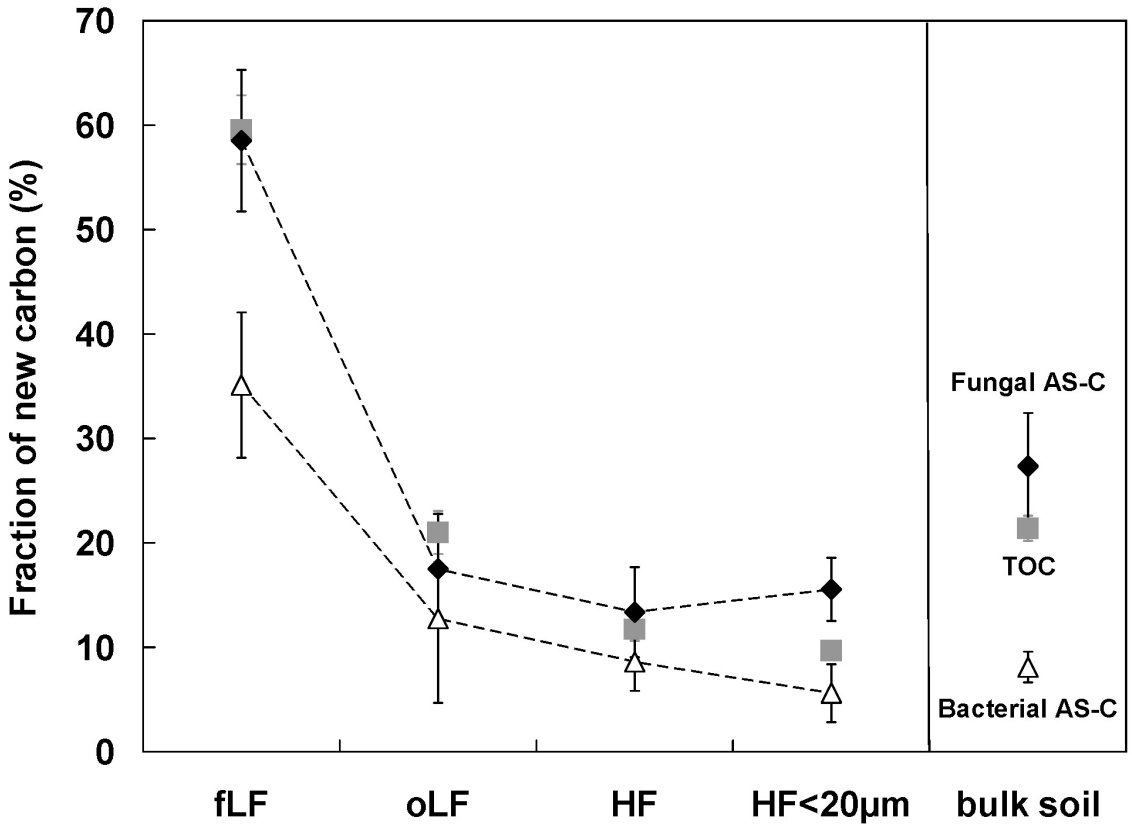

Figure 7 Fraction of new fungal- and bacterial-derived amino sugar carbon (AS-C) as well as total organic carbon (TOC) in bulk soil and soil fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, HF; fine heavy fraction, HF<20µm) after four years of experiment. Average data for the two nitrogen deposition treatments is shown.

The fraction of newly formed fungal amino sugars was in the range of that of total organic C for both, bulk soil and soil fractions (Fig. 7). Generally, the percentage of newly formed fungal amino sugars was higher than that of newly formed bacterial amino sugars in bulk soil and soil fractions (Fig. 7), which indicates that more fungal amino sugars were formed during the experimental period than bacterial amino sugars (Table 2). This is furthermore supported by the soil incubation experiment with isotopically labeled crop residues by Bai *et al.* (2013) showing that glucosamine had shorter mean production times (2.1 – 5.0 days) than galactosamine (2.5 – 9.3 days).

Increased N deposition affected the distribution of both, new (experiment-derived) and old (pre-experimental) amino sugar C in bulk soil and density fractions in several ways. High N treatment significantly increased new, experiment-derived, glucosamine in bulk soil (Table 2) and consequently, also the amount of new fungal-derived C (Fig. 8). Hence, fungal biomass increased after N additions, which is also supported by increased ergosterol concentrations under high N in our experiment (Wiemken *et al.*, 2001a). This contradicts the hypothesis that

increased N deposition leads to a decrease of fungal biomass, due to outcompeting of less efficient fungi that require little N, by high efficient bacteria that are N-limited (Strickland & Rousk, 2010). According to the hypothesis, a shift towards bacteria is expected, when N is not limited, but access to C remains equivalent. The likely reason for the larger amount of new fungal-derived C at high N inputs is the N-induced growth stimulation in our experiment, significantly increasing the fine root biomass and associated ectomycorrhizal fungi (Wiemken *et al.*, 2001a). However, longer-term studies often show that fungal biomass decreases after N additions (Treseder, 2008) and our study may represent an early response to increased N deposition.

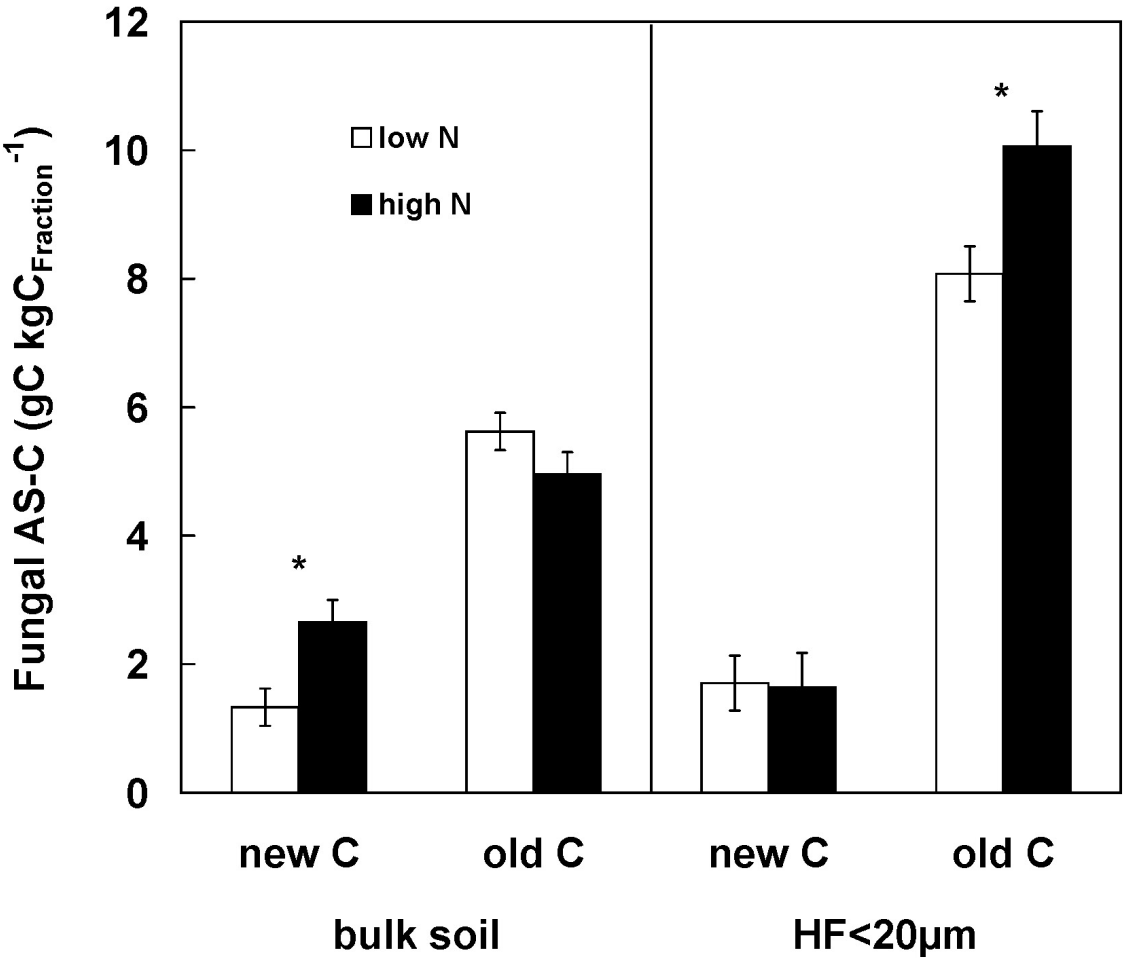

Figure 8 New and old fungal-derived amino sugar carbon (AS-C) in bulk soil and fine heavy fractions (HF<20µm) under low and high nitrogen (N) treatments. Fungal-derived amino sugar carbon was calculated after Van Groenigen *et al.* (2007) and normalized to total organic carbon content in bulk soil and fine heavy fractions, respectively. Asterisks denote significant N treatment effects (* $p<0.05$).

Our results also showed that under increased N deposition, old, pre-experimental glucosamine and galactosamine in fine heavy fractions were significantly higher than in the control (Table 2). Hence, the decomposition of old amino sugar C was reduced under high N additions (Fig. 8). A possible explanation could be that microorganisms are relying less on decomposition of amino sugars, which are rich in N but difficult to decompose, if they receive additional inorganic N. Under high N conditions, microorganisms that need inorganic N to decompose substrates outcompete microorganisms that mine soil organic matter for N (Fontaine *et al.*, 2011). Therefore, mining of soil organic matter increases, when N availability is low. In contrast, mining of soil organic matter is low when N availability is high, which leads to sequestration of C (Fontaine *et al.*, 2011). This mechanism might be especially important in fine heavy fractions, because here organic matter is additionally protected from decomposition by association with soil minerals, which furthermore impedes mining of soil organic matter.

In summary, microbial residues were mainly found in fine heavy fractions, which contained the least amount of new microbial residues. Thus, association with soil minerals seems to be the key process for the stabilization of microbial residues in soil. Furthermore, a consistent decrease of fungal-to-bacterial ratios between free light and fine heavy fractions suggests that bacterial residues are relatively enriched at mineral surfaces compared to fungal residues. In soil fractions and bulk soil, the percentage of new glucosamine was in the range of that of new organic C and more glucosamine was formed during the experimental period compared to galactosamine and muramic acid. Thus, more fungal-derived residues were formed compared to bacterial-derived residues. High N deposition significantly increased new glucosamine in bulk soil and therefore also the amount of new fungal-derived C, which contradicts the hypothesis that, increased N deposition decreases the contribution of fungal-derived residues in soil. Furthermore, old glucosamine and old galactosamine in fine heavy fractions were significantly higher under increased N deposition compared with control. Thus, the decomposition of old microbial residues was reduced under high N deposition, which contradicts the hypothesis that mainly new, experiment-derived, microbial residues are affected as compared to old, pre-experiment, microbial residues. The retarded decomposition of old microbial residues could be due to declined N limitation of microorganisms and therefore reduced dependence on organic N sources. This might be especially important in fine heavy fractions, because of the high interaction and stabilization of microbial residues with soil minerals.

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CHAPTER 4

INTERACTIVE EFFECTS OF ELEVATED CO_2 AND NITROGEN DEPOSITION ON FATTY ACID MOLECULAR AND ISOTOPE COMPOSITION OF ABOVE- AND BELOWGROUND TREE BIOMASS AND FOREST SOIL FRACTIONS

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Author contributions

Frank Hagedorn, Michael W.I. Schmidt and Guido L.B. Wiesenberg proposed the study. Timothy I. Eglinton made the compound-specific stable-isotope-analysis of fatty acids possible and gave conceptual and technical support. Marco Griepentrog conducted the laboratory work, analyzed the data and wrote the manuscript. All authors contributed with constructive comments to the final version of the manuscript.

Abstract

Atmospheric carbon dioxide (CO_2) and reactive nitrogen (N) concentrations have been increasing due to human activities and impact the global carbon (C) cycle by affecting plant photosynthesis and decomposition processes in soil. Large amounts of C are stored in plants and soils, but the mechanisms behind the stabilization of plant- and microbial-derived organic matter (OM) in soils are still under debate and it is not clear how N deposition affects soil OM dynamics. Here, we studied the effects of four years of elevated (^{13}C-depleted) CO_2 and N deposition in forest ecosystems established in open-top chambers on composition and turnover of fatty acids (FAs) in plants and soils. FAs served as biomarkers for plant- and microbial-derived OM in soil density fractions. We analyzed above- and belowground plant biomass of beech and spruce trees as well as soil density fractions for the total organic C and FA molecular and isotope ($\delta^{13}C$) composition.

FAs did not accumulate relative to total organic C in fine mineral fractions, showing that FAs are not effectively stabilized by association with soil minerals. The $\delta^{13}C$ values of FAs in plant biomass increased under high N deposition. However, the N effect was only apparent under elevated CO_2 suggesting a N limitation of the system. In soil fractions, only isotope compositions of short-chain FAs (C_{16+18}) were affected. Fractions of “new” (experimental-derived) FAs were calculated using isotope depletion in elevated CO_2 plots and decreased from free light to fine mineral fractions. “New” FAs were higher in short-chain compared to long-chain FAs (C_{20-30}), indicating a faster turnover of short-chain compared to long-chain FAs. Increased N deposition did not significantly affect the quantity of “new” FAs in soil fractions, but showed a tendency of increased amounts of “old” (pre-experimental) C suggesting that decomposition of “old” C is retarded by high N inputs.

Introduction

Humans are changing the global environment by increasing atmospheric carbon dioxide (CO_2) concentrations and nitrogen (N) deposition, which ultimately leads to perturbations of global biogeochemical cycles and the climate system (Denman *et al.*, 2007). In May 2013, atmospheric CO_2 concentrations reached a landmark of 400 ppm and are still exponentially increasing (Bala, 2013), while atmospheric N deposition increased three- to fivefold within the last century and is expected to further rise in the future (Galloway *et al.*, 2008). Combustion of fossil fuels and fertilizer application are the main sources of reactive N in the atmosphere (Davidson, 2009), which is deposited to terrestrial ecosystems and thereby fertilizes both plant and microbial communities. It is hypothesized that increased N deposition leads to allocation shifts during plant growth from below- to aboveground plant biomass and shifts in microbial community composition from fungal- to microbial-dominated communities (Janssens *et al.*, 2010). The combined effects of elevated CO_2 and N deposition have been shown to affect carbon (C) cycling in plants and soils (Ainsworth & Long, 2005; Reich *et al.*, 2006; Hyvönen *et al.*, 2007; Dieleman *et al.*, 2010; Norby & Zak, 2011).

Soils play a key role in the storage of C in terrestrial ecosystems (Stockmann *et al.*, 2013), but may also release large amounts of C into the atmosphere. It remains unclear how C cycling in the plant-soil system reacts to the environmental changes which are currently underway (Smith, 2012). This is largely due to the fact that the general mechanisms behind stabilization and destabilization of organic matter (OM) in soils are still under debate (Schmidt *et al.*, 2011; Dungait *et al.*, 2012; Cotrufo *et al.*, 2013).

Soil OM is thought to be mainly stabilized by occlusion in soil aggregates and interaction with soil minerals (Sollins *et al.*, 1996; Six *et al.*, 2002; von Lützow *et al.*, 2006). Physical soil fractionation has been frequently used to isolate distinct soil fractions in order to examine different stabilization mechanisms of soil OM (von Lützow *et al.*, 2007). Density fractionation schemes separate bulk soil into “light” fractions, in which OM is either physically unprotected or occluded in aggregates and into “heavy” fractions, in which OM is associated with soil minerals (Golchin *et al.*, 1994; Crow *et al.*, 2007). Although soil density fractions have been frequently analyzed for their total organic C contents (e.g. John *et al.*, 2005; Dorodnikov *et al.*, 2011), the analysis of specific biomarkers in density fractions has only been undertaken in a limited number of studies (Glaser *et al.*, 2000; Wiesenberg *et al.*, 2010a; Griepentrog *et al.*, 2014).

Biomarkers are organic compounds that can be attributed to a specific organism or groups of organisms (Brocks & Pearson, 2005). Ideally, they have the potential to be preserved in any kind of archive such as soils and sediments and can be used as markers for a specific vegetation type, plant part or environment. Therefore, biomarkers can be used as proxies for the source determination of soil OM, such as inputs from plant biomass and microorganisms (Harwood & Russell, 1984; Dinel *et al.*, 1990; Kögel-Knabner, 2002; Amelung *et al.*, 2008).

Fatty acids (FAs) can be used as biomarkers for plant and microbial-derived OM in soils (Dinel *et al.*, 1990). Short-chain FAs ($<C_{20}$) are ubiquitously produced by all living organisms, including plants and microorganisms (Harwood & Russell, 1984). Long-chain FAs ($\geq C_{20}$) are mainly synthesized by plants (Harwood & Russell, 1984) and are therefore frequently used as biomarkers for plant-derived OM in soils (e.g. Bull *et al.*, 2000; Wiesenberg *et al.*, 2004, 2008a, 2012; Otto & Simpson, 2005; Quénéa *et al.*, 2006; Feng *et al.*, 2010). Plant-derived fatty acids are an important energy source for other soil organisms and qualitative or quantitative changes in fatty acid composition can be hypothesized to influence the cycling of organic matter in soils by changing e.g. microbial community structure and decomposition processes. Analyzing the composition and stable isotope signatures of specific biomarkers within distinct soil fractions should greatly enhance our understanding of the turnover and stabilization of OM in soils (Amelung *et al.*, 2008; Feng & Simpson, 2011; Simpson & Simpson, 2012; Gleixner, 2013; Mendez-Millan *et al.*, 2014). The stable isotope analysis of biomarkers in soil fractions is in particular helpful to improve our understanding with respect to different stabilization mechanisms and turnover of plant- and microbial-derived organic matter in soil aggregates and associated with soil minerals.

In this study we used model forest ecosystems that were exposed to ambient and elevated (^{13}C-depleted) CO_2 concentrations in combination with two levels of N deposition. We investigated the molecular and isotope ($\delta^{13}C$) composition of FAs in above- and belowground plant biomass of beech and spruce, as well as in bulk soil and soil density fractions. We tested if elevated CO_2 concentrations and increased N deposition affect the molecular and isotope composition of FAs in above- and belowground plant biomass and soil density fractions. Furthermore, we used the different isotopic signatures of ambient and ^{13}C-depleted CO_2 to isotopically trace the organic matter in different ecosystem compartments. By applying an isotope mixing model, we calculated the fraction of “new” C in soil fractions that is derived from plant inputs throughout the four years of the experimental period. We hypothesized that mainly “new” FAs (produced within the experimental period), are affected by N deposition, in contrast to “old” FAs (that derived from before the experimental period).

Materials and methods

Experimental setup

We used archived plant and soil samples from a combined elevated CO_2 and N deposition experiment that was conducted during four growing seasons between 1994 and 1998. A detailed description of the experimental set-up can be found in Egli *et al.* (1998). In brief, model forest ecosystems were established in open-top chambers with lysimeters: an acidic soil with sandy loamy texture (Haplic Alisol) from a natural beech-spruce forest site was planted with beech (*Fagus sylvatica*) and spruce (*Picea abies*) trees as well as five typical understory plant species (*Carex sylvatica*, *Geum urbanum*, *Hedera helix*, *Ranunculus ficaria*, *Viola sylvatica*). At the start of the experiment, beech trees were two to three years old and spruce trees were four years old.

The model ecosystems were treated with ambient CO_2 (370 µmol mol^{-1}) and elevated CO_2 (570 µmol mol^{-1}) concentrations in combination with low (7 kg NH_4NO_3-N ha^{-1} yr^{-1}) and high (70 kg NH_4NO_3-N ha^{-1} yr^{-1}) levels of N deposition. The isotope value of the elevated CO_2 treatment was depleted in ^{13}C compared to the ambient CO_2 treatment by $\Delta\delta^{13}C$ = 16 ‰ (Hagedorn *et al.*, 2003). The following treatment combinations were studied: ambient CO_2 and low N; ambient CO_2 and high N; elevated CO_2 and low N; elevated CO_2 and high N. Each of the four CO_2 to N treatment combinations was replicated three times in the field.

After four years of treatment, soils were sampled from 0 to 10 cm depth prior to tree harvest. Tree biomass was sampled separately for above- and belowground plant compartments of each tree species including beech leaves, beech roots, spruce needles, spruce roots. All plant and soil samples were dried at 60 °C for 48 h immediately after sampling. Samples were stored in sealed containers in a fully air-conditioned archive (17 °C) with low air humidity (<40 %) until analysis.

Soil fractionation

Soils were physically fractionated following the principle concept of Golchin *et al.* (1994). The fractionation procedure aims at separating three organic matter fractions that conceptually relate to different stabilization mechanisms of organic matter in soil, which cover: organic matter physically unprotected against degradation (i), organic matter protected against degradation by occlusion in soil aggregates (ii) and by association with soil minerals (iii).

Bulk soil was separated into free light fraction (fLF), occluded light fraction (oLF) and total heavy fraction (tHF) using density fractionation along with ultrasonic dispersion. The total

heavy fraction was further separated by particle-size fractionation at 20 µm into a coarse heavy fraction (cHF) and a fine heavy fraction (fHF). The selection of density and ultrasonic dispersion energy was based on preceding experiments to maximize C content in light fractions (Cerli *et al.*, 2012; Griepentrog & Schmidt, 2013). Results showed that a density of 1.6 g cm^{-3} and a dispersion energy of 250 J ml^{-1} were the most suitable parameters for our soil. The fractionation procedure was previously described in detail (Griepentrog *et al.* 2014). In brief, sodium polytungstate (*SPT 0, Tungsten Compounds, Grub am Forst, Germany*) solution was used for density fractionation and SPT solutions were collected and recycled during the fractionation procedure (Six *et al.*, 1999). Bulk soil samples (dried and sieved <2 mm) were suspended in SPT solution and centrifuged. The floating material (fLF) was collected, filtered and rinsed with de-ionized water to remove residual SPT. The remaining soil material was re-suspended in SPT solution and ultrasonically dispersed. The ultrasonic equipment was calorimetrically calibrated according to Schmidt *et al.* (1999). The floating material (oLF) was collected, filtered and rinsed with de-ionized water to remove residual SPT. The remaining soil material (tHF) was rinsed three times with de-ionized water followed by subsequent centrifugation each time. Thereafter, the tHF fraction was particle-size fractionated at 20 µm into cHF and fHF fractions using wet sieving and subsequent sedimentation. All fractions were freeze-dried and milled with a ball mill prior to analysis. All soil samples were fractionated in triplicate and pooled for further analysis. On average, we recovered >99 % of the initial sample masses (Griepentrog *et al.*, 2014).

Bulk carbon and nitrogen analysis

C and N contents and the stable C isotope ratio ($\delta^{13}C$) of plant biomass, bulk soil and soil fractions were determined with an element analyzer coupled to an isotope ratio mass spectrometer (*EA-1110, Carlo Erba, Fisons, Italy*, interfaced with a *ConFlo II* to a *Delta-S, Thermo Finnigan MAT, Bremen, Germany*). Results of the C isotope analysis are expressed in δ units (‰):

$$\delta^{13}C\,(‰) = 1000 \cdot \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \qquad (1)$$

where R = ^{13}C / ^{12}C for both sample and standard. The Vienna Pee Dee Belemnite (VPDB) standard was used as a reference.

Fatty acid analysis

Total lipids were extracted from defined amounts of plant biomass, soil density fractions and bulk soil with a mixture of dichloromethane / methanol (9 / 1; v / v) using microwave extraction for one hour at 1600 W (*MARS Xpress™ Microwave Reaction System, CEM Corporation; cf.* Ernst *et al.,* 2013). After centrifugation, supernatant was collected and residue was washed again with solvent mixture. The procedure was repeated three times to maximize lipid recovery. Total lipid extracts were filtered through glass fiber filters, dried and weighed.

Total lipid extracts were separated into neutral and FA fractions via solid phase extraction using silica gel with 5% potassium hydroxide (*Silica 60 + 5% KOH, 63-200µm, Margot Köhnen-Willsch, Jülich, Germany*). Total lipid extracts were re-dissolved in dichloromethane and transferred to columns filled with the abovementioned silica gel. Neutral lipids were eluted with dichloromethane and collected in round bottom flasks. Thereafter, the FA fraction was eluted with a mixture of dichloromethane / formic acid (99 / 1; v / v) and collected in round flasks. Individual lipid fractions were volume reduced by rotary evaporation, transferred to collection vials and dried thereafter. The FA fractions were derivatized using boron trifluoride / methanol solution (BF_3 / MeOH). Prior to derivatization, deuteriated eicosanoic acid ($C_{20}D_{39}$) was added as an internal standard for quantification.

Compound identification and quantification was performed via an *Agilent 6890 Series* gas chromatograph (GC) coupled to a mass spectrometry detector (*Agilent Technologies, Santa Clara, CA, USA*). GC column was an *Agilent DB-5MS* with 50 m length, 200 µm inner diameter and 0.33 µm film thickness. GC oven temperature program was 50 °C for 2 min, ramp to 150 °C at 5 °C min^{-1}, ramp to 310 °C at 3 °C min^{-1}, hold at 310 °C for 20 min. Carrier gas was Helium flowing at 1 ml min^{-1}.

Compound-specific stable isotope analysis ($\delta^{13}C$) of individual FAs was performed using a *Thermo Trace GC Ultra* connected to a *Thermo Delta V Plus* via a *Thermo GC Isolink* and a *Thermo Conflo 4* (*Thermo Fisher Scientific, Waltham, MA, USA*). GC column was an *Agilent VF-1MS* with 60 m length, 250 µm inner diameter and 0.25 µm film thickness. GC oven temperature program was 45 °C for 1 min, ramp to 130 °C at 40 °C min^{-1}, ramp to 320 °C at 6 °C min^{-1}, hold at 320 °C for 20 min. Carrier gas was Helium flowing at 1 ml min^{-1}. Reproducibility and stability of $\delta^{13}C$ values were evaluated with pulses of CO_2 reference gas and FA methyl ester standards of known isotope composition.

Isotope-based calculations

C isotope ratios ($\delta^{13}C$) of individual FAs were corrected for the $\delta^{13}C$ value of the methyl group that was added during derivatization:

$$\delta_{UD} = \frac{n+1}{n} \cdot \delta_D - \frac{1}{n} \cdot \delta_M \qquad (2)$$

where n is the number of C atoms in the underivatized FA and δ_{UD} and δ_D are the C isotope ratios of the underivatized and the derivatized FAs, respectively. δ_M is the C isotope ratios of the added methyl group (-43.7 ± 0.2). δ_M was determined by repeated measurement of both derivatized and underivatized C_{14} FA and rearrangement of equation 2.

Isotope values of short-chain (C_{16+18}) and long-chain (C_{20-30}) FAs were calculated as the weighted mean of the isotope ratios of the individual FAs ($\overline{\delta}$):

$$\overline{\delta} = \sum_{i=1}^{n} w_i \cdot \delta_i \qquad \text{with: } \sum_{i=1}^{n} w_i = 1 \qquad (3)$$

where w_i is the normalized mass proportion of the individual FAs and δ_i is the isotope ratio of the individual FAs (cf. Wiesenberg *et al.*, 2004, 2008b). Examples of measured isotope ratios of individual FAs of plant biomass and soil fractions can be found in the supporting information (Fig. S1 and S2).

The fraction of new soil C that is derived from plant biomass input during the experimental period was calculated using a simple mixing model (Balesdent *et al.*, 1988):

$$F_{new} = \frac{\delta_{soil,depleted} - \delta_{soil,ambient}}{\delta_{plant,depleted} - \delta_{plant,ambient}} \qquad (4)$$

where $\delta_{soil,depleted}$ and $\delta_{soil,ambient}$ are the $\delta^{13}C$ values of total organic C, short-chain FAs or long-chain FAs in bulk soil or soil fractions for treatments with ^{13}C-depleted CO_2 and ambient CO_2, respectively (cf. Wiesenberg *et al.*, 2008b). Corresponding, $\delta_{plant,depleted}$ and $\delta_{plant,ambient}$ are the $\delta^{13}C$ values of total organic C, short-chain FAs or long-chain FAs in plant biomass for treatments with ^{13}C-depleted CO_2 and ambient CO_2, respectively. For δ_{plant}, mean isotope values of plant biomass (beech leaves, beech roots, spruce needles, spruce roots) were calculated using data from Rasse *et al.* (2005) to estimate the relative distribution of above-

versus belowground plant inputs. Based on Rasse *et al.* (2005), we assumed that after four years of experiment the plant input was divided into 55 % aboveground plant litter and 45 % roots in 0-10 cm soil.

Statistical analysis

The mean is given along with the standard error for replicate measurements (Webster, 2001). Means and standard errors are based on three field replicates. However, due to limited sample amounts, field replicates of plant biomass were pooled for lipid extractions. Here, the means and standard errors are derived from laboratory measurements of the available fractions. Two-way analysis of variance was used to test the significance of treatment effects (N deposition, elevated CO_2) and their interactions (Webster, 2007).

Results

Total organic carbon and total nitrogen

Spruce needles had higher C/N ratios than beech leaves, but spruce roots showed lower C/N ratios than beech roots (Table 1). Only spruce needles were significantly affected by both elevated CO_2 concentration and increased N deposition. Under elevated CO_2, N concentrations decreased and C/N ratios increased. The opposite was true for high N deposition, which increased N concentrations and decreased C/N ratios. Four years continuous addition of ^{13}C-depleted CO_2 ($\Delta\delta^{13}C$ = 16 ‰) led to a significant decrease of $\delta^{13}C$ values of all plant tissues by circa 10 ‰ ($p<0.001$, Table 1). The total organic C isotope values were similar for above- and belowground plant biomass and an identical incorporation of the isotope label was observed for different plant tissues (Fig. 2a).

Soil density fractions showed higher C/N ratios in light than in heavy fractions (Table 2). High N deposition significantly decreased C/N ratios in light fractions. Four years of continuous treatment with ^{13}C-depleted CO_2 significantly decreased $\delta^{13}C$ values of total organic C in all soil fractions and bulk soil ($p<0.001$, Table 2). Heavy fractions revealed higher $\delta^{13}C$ values of total organic C compared to light fractions. Increased N deposition did not affect the $\delta^{13}C$ values of total organic C in plant biomass, soil density fractions and bulk soil (Table 4). More detailed information on the distribution of organic C and total N in soil density fractions and bulk soil of this experiment can be found in Griepentrog *et al.* (2014).

Total lipid and fatty acid composition

No significant effects of elevated atmospheric CO_2 concentration and N deposition were observed for total lipid and FA composition and therefore the means of all four treatments are shown in Fig. 1 and Table 3. Total lipid extract yields were generally higher for plant biomass compared to soil fractions and bulk soil (Table 3). In plant biomass, higher total lipid extract yields were found in above- compared to belowground plant biomass and in spruce compared to beech biomass. In soil density fractions, light fractions had higher total lipid extract yields compared to heavy fractions. The highest total lipid extract yields of all density fractions were found in occluded light fractions, which showed similar values as beech roots (Table 3).

In plant biomass, higher contents of total FAs were found in spruce compared to beech biomass (Fig. 1a). Beech showed similar FA contents in roots and aboveground biomass, while spruce roots had higher FA contents compared to spruce needles. Distinct distribution patterns of FAs were found between roots and aboveground plant biomass. Roots of both tree

species revealed higher contents of methylated and unsaturated FAs compared to aboveground biomass (Fig. 1b).

In soil density fractions, total FA contents decreased in the following order: free light fraction > occluded light fraction > total heavy fraction > fine heavy fraction (Fig. 1a). Distribution patterns of FAs were similar among soil fractions and bulk soil (Fig. 1b). However occluded light fractions had slightly higher contents of dicarboxylic acids compared to the other fractions (Fig. 1b).

Table 1 Organic carbon (C), total nitrogen (N), C/N ratios and stable carbon isotope ratios ($\delta^{13}C$) in plant biomass under low and high N deposition in combination with ambient and elevated CO_2 concentrations. Asterisks denote significant treatment effects: ns = not significant ($p>0.05$), * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

			Carbon	Nitrogen	C / N	$\delta^{13}C$
	N deposition	CO_2 concentration	($gC_{fr}\ kg_{fr}^{-1}$)	($gN_{fr}\ kg_{fr}^{-1}$)	(-)	(‰)
Beech leaves	Low	Ambient	481 ± 4	18.7 ± 0.1	26 ± 0	-30.2 ± 0.2
	Low	Elevated	477 ± 3	18.4 ± 0.1	26 ± 0	-41.8 ± 1.1
	High	Ambient	490 ± 3	19.8 ± 1.2	25 ± 2	-30.7 ± 0.2
	High	Elevated	489 ± 2	19.4 ± 0.6	25 ± 1	-39.5 ± 0.7
Significance	N		**	ns	ns	ns
	CO_2		ns	ns	ns	***
	N x CO_2		ns	ns	ns	ns
Spruce needles	Low	Ambient	497 ± 0	8.0 ± 0.1	62 ± 1	-30.2 ± 0.1
	Low	Elevated	496 ± 1	6.8 ± 0.1	74 ± 1	-41.5 ± 0.9
	High	Ambient	503 ± 3	10.4 ± 0.6	49 ± 3	-30.1 ± 0.0
	High	Elevated	494 ± 6	9.1 ± 0.0	55 ± 1	-40.3 ± 0.6
Significance	N		ns	***	***	ns
	CO_2		ns	**	**	***
	N x CO_2		ns	ns	ns	ns
Beech roots	Low	Ambient	491 ± 4	7.1 ± 1.0	73 ± 12	-28.9 ± 0.2
	Low	Elevated	461 ± 14	7.7 ± 1.1	64 ± 13	-41.0 ± 1.2
	High	Ambient	468 ± 11	8.5 ± 1.9	62 ± 16	-29.1 ± 0.1
	High	Elevated	474 ± 10	7.3 ± 0.5	66 ± 3	-39.0 ± 0.6
Significance	N		ns	ns	ns	ns
	CO_2		ns	ns	ns	***
	N x CO_2		ns	ns	ns	ns
Spruce roots	Low	Ambient	483 ± 7	9.8 ± 0.6	50 ± 3	-29.1 ± 0.2
	Low	Elevated	453 ± 7	9.2 ± 0.2	49 ± 1	-39.1 ± 0.8
	High	Ambient	459 ± 13	11.2 ± 0.6	41 ± 3	-28.7 ± 0.1
	High	Elevated	449 ± 12	9.3 ± 0.2	48 ± 1	-40.2 ± 1.9
Significance	N		ns	ns	ns	ns
	CO_2		ns	*	ns	***
	N x CO_2		ns	ns	ns	ns

Table 2 Organic carbon (C), total nitrogen (N), C/N ratios and stable carbon isotope ratios ($\delta^{13}C$) in soil density fractions and bulk soil under low and high N deposition in combination with ambient and elevated CO_2 concentrations. Asterisks denote significant treatment effects: ns = not significant ($p>0.05$), * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

	N deposition	CO_2 concentration	Carbon ($gC_{fr}\ kg_{fr}^{-1}$)	Nitrogen ($gN_{fr}\ kg_{fr}^{-1}$)	C / N (-)	$\delta^{13}C$ (‰)
Free light fraction (fLF)	Low	Ambient	321 ± 7	9.9 ± 0.1	33 ± 0	-29.1 ± 0.2
	Low	Elevated	326 ± 7	9.7 ± 0.3	34 ± 2	-35.4 ± 0.5
	High	Ambient	323 ± 14	11.8 ± 0.4	28 ± 1	-29.1 ± 0.1
	High	Elevated	330 ± 5	11.4 ± 0.1	29 ± 1	-35.4 ± 0.4
Significance	N		ns	***	**	ns
	CO_2		ns	ns	ns	***
	N x CO_2		ns	ns	ns	ns
Occluded light fraction (oLF)	Low	Ambient	394 ± 16	12.5 ± 0.2	31 ± 1	-28.7 ± 0.0
	Low	Elevated	393 ± 16	11.8 ± 0.2	33 ± 1	-30.9 ± 0.4
	High	Ambient	349 ± 17	12.1 ± 0.3	29 ± 1	-28.5 ± 0.1
	High	Elevated	357 ± 6	12.7 ± 0.3	28 ± 0	-30.7 ± 0.2
Significance	N		*	ns	***	ns
	CO_2		ns	ns	ns	***
	N x CO_2		ns	*	ns	ns
Total heavy fraction (tHF)	Low	Ambient	9.6 ± 0.1	0.7 ± 0.0	13 ± 0	-26.0 ± 0.0
	Low	Elevated	9.3 ± 0.1	0.7 ± 0.0	13 ± 0	-27.1 ± 0.1
	High	Ambient	9.5 ± 0.2	0.8 ± 0.0	13 ± 0	-26.0 ± 0.1
	High	Elevated	9.9 ± 0.3	0.8 ± 0.0	13 ± 0	-27.4 ± 0.1
Significance	N		ns	**	*	ns
	CO_2		ns	ns	ns	***
	N x CO_2		ns	ns	ns	ns
Fine heavy fraction (fHF)	Low	Ambient	23.6 ± 0.3	2.2 ± 0.1	11 ± 0	-26.3 ± 0.0
	Low	Elevated	22.2 ± 0.7	1.9 ± 0.1	12 ± 0	-27.2 ± 0.1
	High	Ambient	22.9 ± 0.3	2.0 ± 0.0	11 ± 0	-26.2 ± 0.0
	High	Elevated	23.7 ± 0.4	2.1 ± 0.0	11 ± 0	-27.3 ± 0.1
Significance	N		ns	ns	ns	ns
	CO_2		ns	ns	ns	***
	N x CO_2		*	**	ns	ns
Bulk soil	Low	Ambient	15.1 ± 1.0	1.0 ± 0.0	16 ± 0	-26.9 ± 0.2
	Low	Elevated	14.7 ± 0.6	1.0 ± 0.1	15 ± 0	-29.1 ± 0.1
	High	Ambient	14.3 ± 0.8	1.0 ± 0.0	15 ± 0	-27.0 ± 0.1
	High	Elevated	15.1 ± 0.2	1.0 ± 0.0	15 ± 0	-29.2 ± 0.2
Significance	N		ns	ns	*	ns
	CO_2		ns	ns	ns	***
	N x CO_2		ns	ns	ns	ns

Mass distribution between soil fractions: fLF = 0.9 ± 0.02 %, oLF = 0.2 ± 0.01 %, tHF = 98.9 ± 0.1 %, fHF = 31.1 ± 0.2 %

Carbon distribution between soil fractions: fLF = 21.8 ± 0.6 %, oLF = 6.9 ± 0.2 %, tHF = 71.3 ± 1.0 %, fHF = 62.6 ± 1.0 %

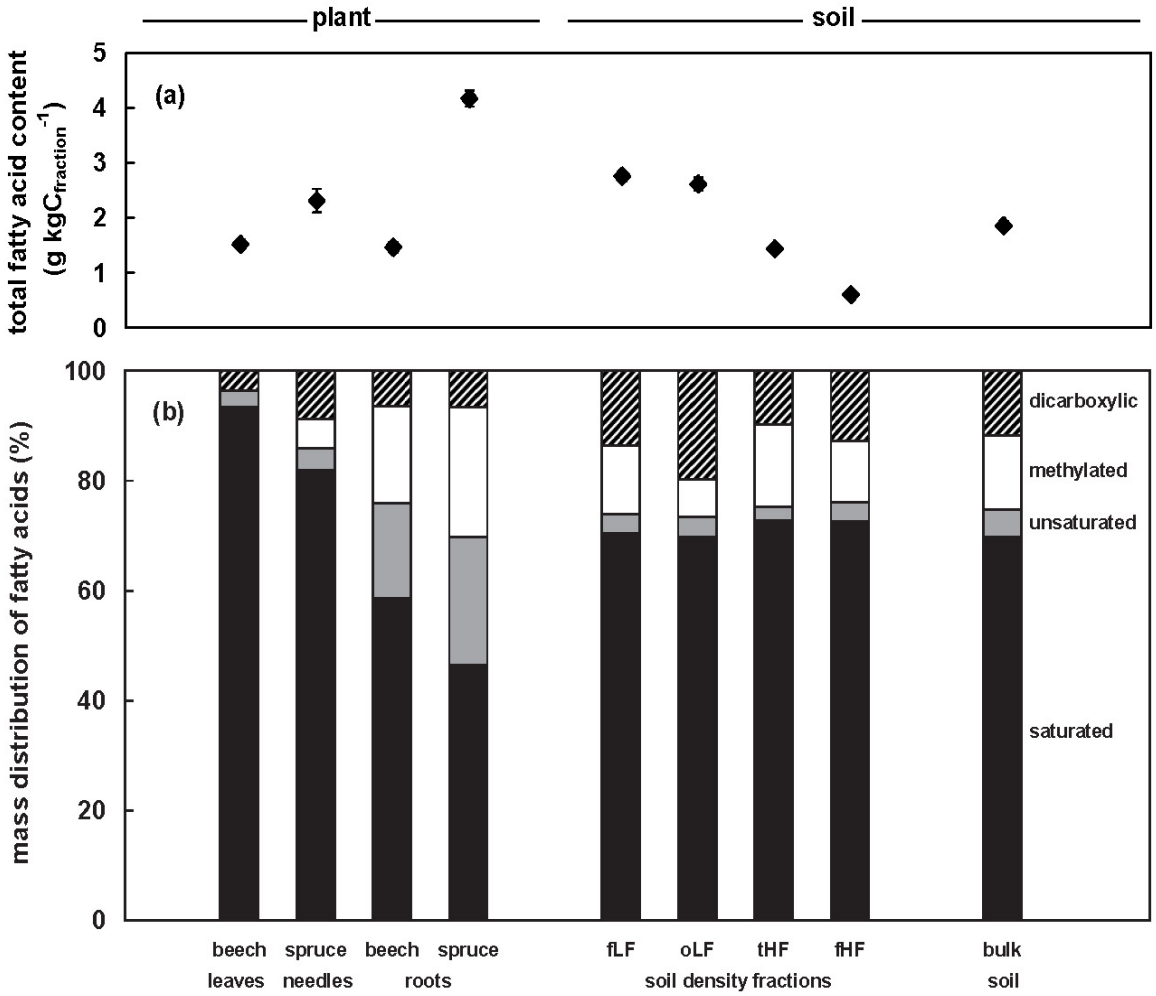

Figure 1 Total fatty acid content (a) and mass distribution of fatty acids (b: saturated, unsaturated, methylated, dicarboxylic fatty acids) in plant biomass, soil density fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, tHF; fine heavy fraction, fHF) and bulk soil. Mean of all four treatments is shown.

Fatty acid molecular proxies

Several molecular proxies have been proposed to assess the source of fatty acids in soils at the molecular level (Wiesenberg *et al.*, 2010a). The ratio of unsaturated-to-saturated C_{16} FAs ($C_{16:1}$ / $C_{16:0}$) is generally higher in microbial- compared to plant-derived OM, due to the common presence of $C_{16:1}$ FAs in microbial tissue (Harwood & Russell, 1984). Furthermore, unsaturated C_{18} FAs mainly derive from plant biomass and only partially from microorganisms (Harwood & Russell, 1984). Consequently, high ratios of unsaturated-to-saturated C_{18} FAs ($C_{18:1-2}$ / $C_{18:0}$) indicate fresh, mainly plant-derived OM and decreasing values reflect ongoing degradation from plant biomass towards soil OM (Wiesenberg *et al.*, 2010a).

No significant effects of CO_2 concentrations and N deposition on FA molecular proxies were observed and therefore the means of all four treatments are shown in Table 3. In plant biomass, higher ratios of unsaturated-to-saturated C_{16} FAs were determined in roots compared to aboveground plant tissues and in spruce compared to beech biomass (Table 3). The ratio of unsaturated-to-saturated C_{18} FAs was one order of magnitude higher in below- compared to aboveground plant biomass. In soil fractions, the ratio of unsaturated-to-saturated C_{16} FAs increased from free light towards fine heavy soil fractions, while the ratio of unsaturated-to-saturated C_{18} FAs decreased in the same order (Table 3).

Average chain lengths can be used as molecular proxy for the source of FAs (Wiesenberg *et al.*, 2010a). Microbial-derived FAs are characterized by lower average chain lengths than plant-derived FAs, due to the absence of any long-chain fatty acids (>C_{19}). In plant biomass, lower average chain lengths were found in roots compared to aboveground plant biomass (Table 3). In soil density fractions, occluded light fractions revealed extreme low values of long-chain versus short-chain FAs (0.4 ± 0.0) as well as average chain lengths (18.7 ± 0.1) compared to other soil fractions and bulk soil.

Table 3 Total lipid extract yield and fatty acid molecular proxies in plant biomass, soil density fractions and bulk soil. Molecular proxies: unsaturated to saturated C_{16} fatty acids ($C_{16:1}$ / $C_{16:0}$); unsaturated to saturated C_{18} fatty acids ($C_{18:1-2}$ / $C_{18:0}$); long-chain to short-chain fatty acids (LC / SC); average chain length (ACL). Mean of all four treatments is shown.

	Lipid extract yield		Fatty acid molecular proxies			
	(mg kg^{-1})	(g kgC^{-1})	$C_{16:1}$ / $C_{16:0}$	$C_{18:1-2}$ / $C_{18:0}$	LC / SC	ACL
Plant materials						
Beech leaves	66574 ± 3831	166 ± 10	0.03 ± 0.00	0.3 ± 0.0	1.0 ± 0.0	20.8 ± 0.1
Spruce needles	132300 ± 5956	331 ± 15	0.14 ± 0.02	0.3 ± 0.1	1.5 ± 0.1	21.0 ± 0.1
Beech roots	28011 ± 1808	70 ± 5	0.09 ± 0.01	2.8 ± 0.5	1.2 ± 0.1	19.6 ± 0.1
Spruce roots	41878 ± 2660	105 ± 7	0.20 ± 0.04	3.9 ± 0.2	1.7 ± 0.1	19.9 ± 0.1
Soil density fractions						
Free light fraction (fLF)	21718 ± 671	67 ± 2	0.02 ± 0.00	0.5 ± 0.1	1.1 ± 0.1	20.4 ± 0.1
Occluded light fraction (oLF)	28182 ± 932	75 ± 2	0.05 ± 0.01	0.3 ± 0.0	0.4 ± 0.0	18.7 ± 0.1
Total heavy fraction (tHF)	338 ± 9	35 ± 1	0.04 ± 0.00	0.3 ± 0.0	1.7 ± 0.1	21.4 ± 0.2
Fine heavy fraction (fHF)	770 ± 21	33 ± 1	0.12 ± 0.01	0.2 ± 0.0	1.5 ± 0.0	20.6 ± 0.1
Bulk soil	610 ± 16	41 ± 1	0.07 ± 0.01	0.7 ± 0.1	1.4 ± 0.1	21.1 ± 0.2

LC / SC = ΣC_{20-32} / ΣC_{16-19} ; ACL = $\Sigma(z_n \times n) / \Sigma(z_n)$

Carbon isotope composition ($\delta^{13}C$) of fatty acids

Similar to what we found for total organic C, the treatment with ^{13}C-depleted CO_2 significantly decreased the $\delta^{13}C$ values of FAs in plant biomass, soil density fractions and bulk soil, compared to control samples (Table 4, $p<0.001$).

Short-chain FAs (C_{16+18}) in roots were characterized by higher $\delta^{13}C$ values compared to aboveground plant biomass under both ambient and elevated CO_2 concentrations (Fig. 2b). The $\delta^{13}C$ values of short-chain FAs in light soil fractions revealed similar values to those of roots biomass for ambient and elevated CO_2 concentrations. The $\delta^{13}C$ values of short-chain FAs increased in the following order in soil fractions under both CO_2 concentrations: free light fraction < occluded light fraction < total heavy fraction < fine heavy fraction. Increased N deposition affected the C isotope composition of short-chain FAs in plant biomass, soil density fractions and bulk soil, except for the fine heavy soil fraction, which was not affected by increased N deposition (Table 4). However, there was a statistically significant interaction between increased N deposition and elevated CO_2 concentrations, except for spruce roots and fine heavy fractions. Short-chain FAs were only affected by increased N deposition under elevated CO_2 concentrations, leading to higher $\delta^{13}C$ values. N deposition effects were larger in beech compared to spruce biomass.

Long-chain FAs (C_{20-30}) did not show distinct differences between the $\delta^{13}C$ values of root and aboveground plant biomass for both ambient and elevated CO_2 concentrations (Fig. 2c) in contrast to short-chain FAs (Fig. 2b). Similar to short-chain FAs, the $\delta^{13}C$ values of long-chain FAs increased in the following order in soil fractions: free light fraction < occluded light fraction < total heavy fraction < fine heavy fraction (Fig. 2c). However, the increase was less pronounced under ambient compared to elevated CO_2. Increased N deposition significantly affected the C isotope composition of long-chain FAs in plant biomass, except for spruce roots (Table 4). As observed for short-chain FAs, long-chain FAs also revealed a significant interaction between N deposition and atmospheric CO_2concentration. Increased N deposition only showed significant effects under elevated CO_2 concentration, thereby leading to higher $\delta^{13}C$ values of long-chain FAs (Fig. 2c). In contrast to short-chain FAs, long-chain FAs were not affected by increased N deposition in soil density fractions and bulk soils (Table 4).

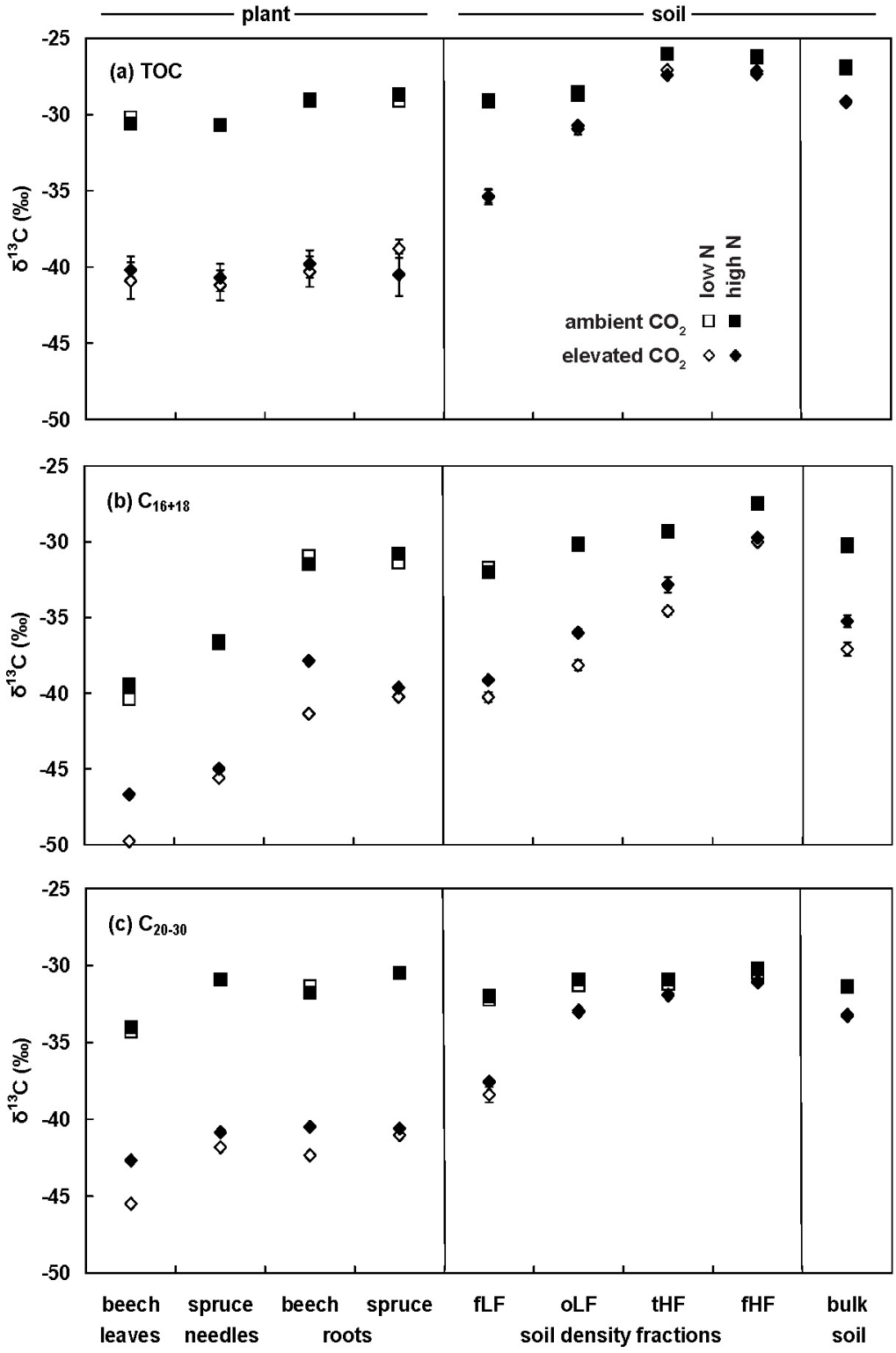

Figure 2 Carbon isotope ratios ($\delta^{13}C$) of total organic carbon (TOC, a), short-chain (C_{16+18}, b) and long-chain (C_{20-30}, c) fatty acids in plant biomass, soil density fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, tHF; fine heavy fraction, fHF) and bulk soil. Ambient and elevated CO_2 concentrations were applied in combination with low and high nitrogen (N) deposition for four years. Note that elevated CO_2 treatment was depleted in ^{13}C by 16‰ compared to ambient CO_2.

Estimation of new and old fatty acids in soil density fractions and bulk soils

For both short-chain (C_{16+18}) and long-chain (C_{20-30}) FAs in soils, fractions of new experimental-derived FA C decreased in the following order: free light fraction > occluded light fraction > total heavy fraction > fine heavy fraction (Fig. 3). Within all soil density fractions and bulk soil, long-chain FAs showed approximately equal fractions of new C compared with total organic C, while short-chain FAs generally showed significantly higher fractions of new C (Fig. 3). Although the fraction of new C is lower in long-chain compared to short-chain FAs, higher total amounts of new C were found in long-chain FAs (Fig. 4). However, in contrast to other soil fractions and bulk soil, occluded light fractions showed higher (double) amounts of short-chain compared to long-chain FAs (Fig. 4). Increased N deposition had no significant effects on the fractions of new C in short-chain and long-chain FAs in all soil fractions and bulk soil (Fig. 3). Also the total amounts of new short-chain and long-chain FAs were not affected by increased N deposition (Fig. 4). In contrast to new FA C, the amounts of old long-chain FA C were 7.8 % higher in fine heavy fractions and 15.4 % higher in bulk soil under increased N deposition compared to the control (Fig. 4). However, the effect only tended to be significant ($p = 0.094$ for fine heavy fractions; $p = 0.075$ for bulk soil).

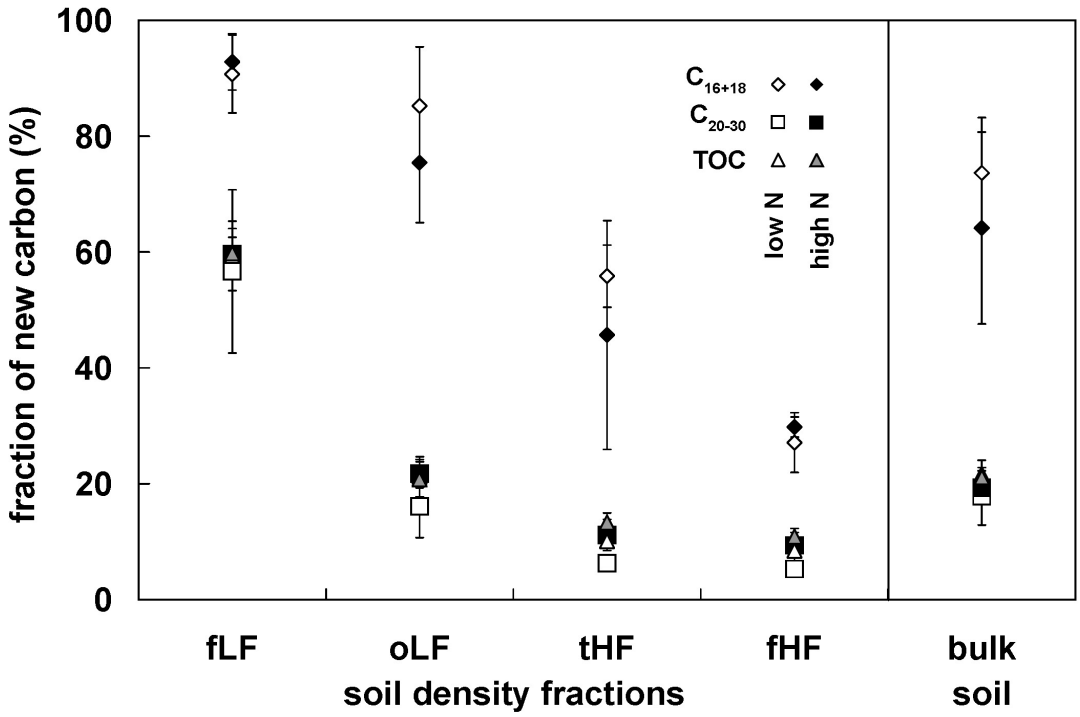

Figure 3 Fraction of new short-chain (C_{16+18}) and long-chain (C_{20-30}) fatty acid carbon and total organic carbon (TOC) in soil density fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, tHF; fine heavy fraction, fHF) and bulk soil after four years of treatment with low and high nitrogen (N) deposition.

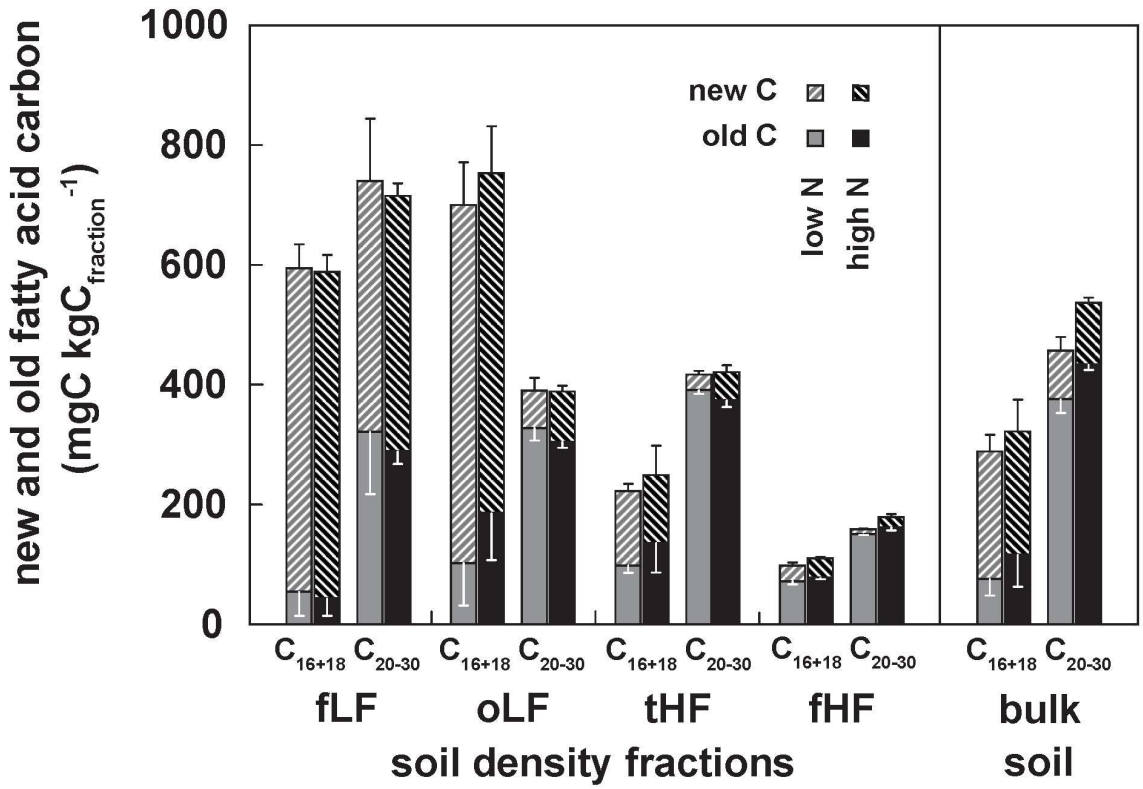

Figure 4 New and old short-chain (C_{16+18}) and long-chain (C_{20-30}) fatty acid carbon in soil density fractions (free light fraction, fLF; occluded light fraction, oLF; total heavy fraction, tHF; fine heavy fraction, fHF) and bulk soil after four years of treatments with low and high nitrogen (N) deposition.

Table 4 Level of significance of increased nitrogen (N) deposition and elevated (^{13}C-depleted) CO_2 treatment effects and their interaction (N × CO_2) on $\delta^{13}C$ values of short-chain (C_{16+18}) and long-chain (C_{20-30}) fatty acids in plant biomass, soil density fractions and bulk soil. Asterisks denote level of significance: ns = not significant (p>0.05), * p<0.05, ** p<0.01, *** p<0.001.

	Number of replicates		Short-chain fatty acids (C_{16+18})			Long-chain fatty acids (C_{20-30})		
	Field	Analytical	N	CO_2	N x CO_2	N	CO_2	N x CO_2
Plant materials								
Beech leaves	1	4	***	***	***	***	***	***
Spruce needles	1	4	*	***	**	***	***	***
Beech roots	1	4	***	***	***	***	***	***
Spruce roots	1	4	**	***	ns	ns	***	ns
Soil density fractions								
Free light fraction (fLF)	3	4	*	***	***	ns	***	ns
Occluded light fraction (oLF)	3	4	***	***	***	ns	***	ns
Total heavy fraction (tHF)	3	4	**	***	**	ns	***	ns
Fine heavy fraction (fHF)	3	4	ns	***	ns	ns	***	ns
Bulk soil	3	4	*	***	**	ns	***	ns

Discussion

Bulk carbon and nitrogen

In plant biomass, C/N ratios varied between different plant tissues and species (Table 1), which has been commonly reported (e.g. Otto & Simpson, 2005). Elevated atmospheric CO_2 concentration and increased N deposition influenced the N contents of spruce needles in opposite directions. While high N deposition increased N contents, elevated CO_2 decreased N contents, with corresponding changes most evident in the C/N ratio of spruce needles (Table 1). Contrasting effects of elevated CO_2 concentration and N deposition on the C and N composition of plant biomass have been observed previously (Saxe *et al.*, 1998; Hyvönen *et al.*, 2007) and are attributed to changes in photosynthesis (Ainsworth & Long, 2005) or OM allocation within the plant (Dieleman *et al.*, 2010). The overall change of plant isotope composition ($\delta^{13}C$) by circa 10 ‰ due to four years of isotope labeling is similar to other free air CO_2 enrichment experiments (e.g. Van Kessel *et al.*, 2000).

In soil density fractions, high C/N ratios in light fractions reflect C/N ratios of plant biomass input, while lower C/N ratios in heavy fractions are attributed to an increased contribution of microbial-derived OM in mineral soil fractions (Golchin *et al.*, 1994). N effects on C/N ratios in light fractions are consistent with effects observed for plant biomass, which is the main constituent of OM in light fractions (Crow *et al.*, 2007; Wagai *et al.*, 2009; Dorodnikov *et al.*, 2011). An extended discussion on the distribution of C and N in soil fractions of this experiment can be found in Griepentrog *et al.* (2014).

Total lipid and fatty acid composition

Total lipid extract yields varied between different plant tissues and soil fractions (Table 3) and are in the range of values found in density fractions of a cropland soil (Wiesenberg *et al.*, 2010a). Contents and distribution patterns of FA varied between the two tree species in our experiment (Fig. 1), which has also been observed for other plant species in forests (Otto & Simpson, 2005; Feng *et al.*, 2010), in grassland (Wiesenberg *et al.*, 2008a) as well as in cropland (Wiesenberg *et al.*, 2004). Spruce roots showed significantly higher FA contents (Fig. 1) and lower average chain lengths than spruce needles (Table 3) and short-chain FAs of microbial origin might therefore be an additional source of root FAs (Harwood & Russell, 1984; Lichtfouse *et al.*, 1995). In contrast to aboveground biomass, roots are generally associated with microbial communities, in particular mycorrhizal fungi (Jones *et al.*, 2009) and might be therefore analyzed together. High amounts of methylated (Fig. 1) and mono-

unsaturated (Table 3) FAs in root biomass furthermore point towards a considerable contribution of microbial-derived FAs in roots (Harwood & Russell, 1984).

Distribution patterns of FAs were similar among soil fractions and bulk soil (Fig. 1) and most likely reflect a mixed contribution of root, aboveground plant and rhizomicrobial FAs (Wiesenberg *et al.,* 2012; Mueller *et al.,* 2013). The decrease of FA contents from free light to fine heavy fractions (Fig. 1) might be due to high inputs of fresh plant-derived FAs into light fractions and a trend of increasing degradation towards minerals soil fractions. This is also consistent with the decrease of mainly plant-derived polyunsaturated C_{18} FAs from free light towards fine heavy fractions (Table 3). Low values of long-chain versus short-chain FA ratios and average chain lenghts in occluded light fractions (Table 3) point towards a high contribution of microbial-derived FAs, which is consistent with the prominent role of microorganisms in soil aggregate formation (Six & Paustian, 2014). Our results are in general agreement with those from a study of FAs in density fractions of a cropland soil (Wiesenberg *et al.,* 2010a). However, the lack of FA accumulation relative to total organic C in fine mineral fractions (Fig. 1) contradicts observations made by Griepentrog *et al.* (2014) for microbial-derived amino sugars in this experiment. This suggests that FAs are not as effectively stabilized by association with soil minerals compared to microbial sugars or total organic C, which might be related to the fast incorporation of FAs in soils (Wiesenberg *et al.*, 2010b).

Carbon isotope composition ($\delta^{13}C$) of fatty acids

Short-chain FAs of roots were characterized by higher $\delta^{13}C$ values compared to aboveground biomass (Fig. 2b). Root C is derived from photosynthetically fixed C that was translocated within the plant (Hobbie & Werner, 2004). Metabolic intermediates (such as sugars) are fixed and released several times during transport to roots and then synthesized into root FAs (Hobbie & Werner, 2004), which causes isotope fractionation and mainly leads to the ^{13}C enrichment of short-chain FAs in roots (Wiesenberg *et al.,* 2004). Additionally, microorganisms are generally associated with plant roots (Jones *et al.,* 2009) and typically enriched in ^{13}C compared to plant-derived OM, due to isotope fractionation during microbial uptake and biochemical transformation (Werth & Kuzyakov, 2010). Therefore, microbial-derived short-chain FAs could potentially also contribute to the observed ^{13}C enrichment in roots. However, root samples were thoroughly washed before extraction and analysis and microbial remains should only be present in minor quantities, mainly as microorganisms living within the root tissues.

Short-chain FAs in light soil fractions revealed similar $\delta^{13}C$ values to those in root biomass (Fig. 2b). Therefore, root-derived OM may contribute substantial quantities to plant-derived inputs of soil FAs (Wiesenberg *et al.*, 2008b, 2012), as suggested for total soil organic C (Rasse *et al.*, 2005). However, it is also possible that OM in light fractions might be derived from aboveground plant biomass that was altered during microbial degradation and had therefore undergone isotope fractionation (Nguyen Tu *et al.*, 2004). The latter would also account for the progressive enrichment in ^{13}C from free light towards fine heavy soil fractions.

In plant biomass, isotope compositions of both short-chain (C_{16+18}) and long-chain (C_{20-30}) FAs were significantly affected by increased N deposition (Table 4). However, N deposition effects only occurred under elevated CO_2 concentrations, showing significant interactions between CO_2 concentrations and N deposition (Table 4). The combined effects of increased N deposition and elevated CO_2 concentrations may be related to changes in C isotope fractionation during photosynthesis (Huang *et al.*, 1999). Under elevated atmospheric CO_2 concentration the ratio of partial pressures of CO_2 concentrations within and outside the leaves changes, resulting in stomatal closure (Ainsworth & Rogers, 2007). However, N application stimulates plant growth and promotes photosynthesis, which increases the consumption of intercellular CO_2 and reduces intercellular CO_2 partial pressure (Huang *et al.*, 1999). The combined effects of reduction in stomatal conductance and increase in intercellular CO_2 consumption lead to a reduction of ^{13}C discrimination during photosynthesis and therefore to higher $\delta^{13}C$ values (Farquhar *et al.*, 1989).

The observed isotope effects were larger for beech compared with spruce biomass (Fig 2b, c). Beech grows better on sites with high contents of available nutrients and high pH values, while spruce is more adapted to sites that are depleted in nutrients and characterized by low pH values (Spinnler *et al.*, 2002). Also for our experiment it could be shown that beech was more dependent on N availability than spruce (Hagedorn *et al.*, 2002).

In soil fractions, only $\delta^{13}C$ values of short-chain FAs were affected by increased N deposition. Here, N effects can be attributed to changes in $\delta^{13}C$ values of plant biomass input, but probably also to N effects on microbial-derived FAs. The latter might be more important since no effects on plant-specific long-chain FAs could be observed in soil fractions (Fig 2c, Table 4). Furthermore, measurements of amino sugars as biomarkers for microbial residues support this assumption, since they indicated higher production of fungal residues under increased N deposition (Griepentrog et al., 2014). Therefore, our results suggest that N effects in soil fractions are mainly attributed to microorganisms and only in minor parts to changes in plant-

derived OM composition, which was recently also suggested for soil OM genesis (Miltner *et al.*, 2012).

New and old fatty acid carbon in soil fractions

Fractions of new FA C decreased from free light towards fine heavy soil fractions (Fig. 3) reflecting higher inputs of isotopically-labeled plant biomass into light fractions. In heavy fractions, organic matter was altered by microorganisms, causing isotope fractionation (^{13}C enrichment) as part of the microbial loop (Bonkowski, 2004; Hobbie & Werner, 2004), which in turn led to less incorporation of new C in mineral fractions (Fig. 2).

While long-chain FAs revealed equal fractions of new C compared with total organic C, short-chain FAs had significantly higher fractions of new C. Long-chain FAs are indicative for plant biomass, which substantially contributes to soil OM inputs and total organic C, and they are therefore expected to have similar fractions of new C. However, in previous studies it has also been reported that FAs have faster turnover times than total organic C (Wiesenberg *et al.*, 2004; Wiesenberg *et al.*, 2008b; Feng *et al.*, 2010). This indicates that, in our experiment, also other compounds with similar turnover times to those of long-chain FAs have largely been incorporated into soil OM or that the comparatively short-term duration of the experiments of four years might have biased the results.

Short-chain FAs show higher contributions of new C compared to long-chain FAs throughout all soil fractions (Fig. 3), which confirms prior observations in grassland soils (Wiesenberg *et al.*, 2008b). The main reason is the additional contribution of microorganisms to short-chain FAs and the faster incorporation of the isotope label into microbial-derived OM with a shorter turnover compared to plant biomass (Kramer & Gleixner, 2006). An additional process that could have partially contributed to the observed pattern is the rapid incorporation of plant-derived short-chain FAs incorporated via root exudates (Wiesenberg *et al.*, 2010b).

Although increased N deposition altered the isotope composition of FAs (Fig. 2), N deposition did not significantly affect the fraction of new FA C in soil fractions and bulk soil (Fig. 3). The absence of significant effects might be attributed to large errors associated with the analysis, the calculation of new C fractions and the duration of the experiment of only 4 years. However, there is a (statistically insignificant) trend that high N deposition decreased the fraction of new C in short-chain FAs (by 10%) in occluded light and total heavy fractions as well as in bulk soil (Fig. 3). This could be attributed to N effects on microorganisms, leading to a decline in microbial biomass under increased N deposition (Treseder, 2008).

Amounts of old FA C were 7.8 % ($p = 0.094$) and 15.4 % ($p = 0.075$) higher in fine heavy fractions and bulk soil respectively, under high N deposition compared to control treatments. We attribute the weak significance level to the short duration of the experiment, the large stocks of C in soil and high uncertainty in calculating fractions of new and old C (Fig. 3, 4). However, the higher amounts of old C in FAs are in agreement with other findings within the same experiment, showing that there is a retarded decomposition of old total organic C (Hagedorn *et al.*, 2003) and old microbial residue C (Griepentrog *et al.*, 2014) under high N deposition. The decrease in decomposition of old C under high N deposition could be attributed to reduced mining of native soil OM by microorganisms, if additional inorganic N is available (Fontaine *et al.*, 2011). This process seems to be especially important in fine heavy fractions where OM is associated with soil minerals.

In summary, we were able to trace new versus old above- and belowground plant-derived FAs into forest soil density fractions using compound-specific stable isotope analysis. Our results suggest a substantial contribution of root-derived FAs to the soil FA pool. In contrast to total organic C, FAs are apparently not preferentially stabilized by association with soil minerals. N deposition increased $\delta^{13}C$ values of plant FAs under elevated CO_2 concentrations, most likely due to lesser isotope discrimination during photosynthesis. However, in soil fractions only short-chain FAs were affected by increased N deposition, which could be related to a faster turnover throughout all soil fractions compared to plant-derived long-chain FAs. The absolute quantities of new C in FAs of soil density fractions and bulk soil were not significantly affected, at least partly due to the short experimental period of four years. However, we found a tendency of increasing amounts of old FA-C under high N inputs which is in agreement with previous findings that N deposition retards the decomposition of old soil C. Temperate forest ecosystems are the major ecosystems in Europe and the retarded decomposition of native soil OM under increased N deposition might affect the C balance of European soils, because it potentially increases soil C sequestration. However, at a global scale, the study of boreal and tropical forest ecosystems is a potential area of future research with high relevance to global soil C storage.

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PUBLICATIONS AND PRESENTATIONS

Publications in peer-reviewed journals

Griepentrog, M., Eglinton, T.I., Hagedorn, F., Schmidt, M.W.I., Wiesenberg, G.L.B., 2014. Interactive effects of elevated CO_2 and nitrogen deposition on fatty acid molecular and isotope composition of above- and belowground tree biomass and forest soil fractions. *Global Change Biology*, doi:10.1111/gcb.12666.

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Griepentrog, M., Bodé, S., Boeckx, P., Hagedorn, F., Wiesenberg, G.L.B., Schmidt, M.W.I., 2013. No effects of increased nitrogen deposition on the composition and turnover of plant and microbial biomarkers in forest soil density fractions. (Presentation at the *26th International Meeting on Organic Geochemistry (IMOG)*, September 2013 in Tenerife, Spain)

Griepentrog, M., Bodé, S., Boeckx, P., Hagedorn, F., Wiesenberg, G.L.B., Schmidt, M.W.I., 2013. Increased nitrogen deposition did not affect the composition and turnover of plant and microbial biomarkers in forest soil density fractions. (Presentation at the *General Assembly of the European Geosciences Union (EGU)*, April 2013 in Vienna, Austria)

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Griepentrog, M., Bodé, S., Boeckx, P., Schmidt, M.W.I., 2012. Amino sugar dynamics in forest soil exposed to increased nitrogen deposition – Composition and turnover in soil density fractions. (Presentation at the *General Assembly of the European Geosciences Union (EGU)*, April 2012 in Vienna, Austria)

Griepentrog, M., Heim, A., Hagedorn, F., Smittenberg, R.H., Schmidt, M.W.I., 2011. Impact of increased nitrogen deposition on the turnover of plant- and microbially-derived organic matter compounds in density fractions of forest soils. (Presentation at the *Biannual Meeting of the German Soil Science Society (DBG)*, September 2011 in Berlin, Germany)

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